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Identification of genetic markers predictive of paclitaxel-induced peripheral neuropathy

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A mis padres, a mis abuelos y a David

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ABSTRACT

Paclitaxel, an antimitotic agent widely used for the treatment of solid tumors, has the neuropathy as its dose limiting toxicity. In the majority of the patients the neuropathy resolves after finishing the treatment; however, in severe cases the nerves can be irreversibly damaged affecting permanently patients' quality of life. In addition, neuropathy-induced dose reductions and treatment suspensions are common and may lead to sub-optimal disease treatment and increased likelihood of relapse. The individual genetic background has been suggested to explain a substantial proportion of the inter-individual variability observed in paclitaxel toxicites. Thus, the main goal of this Thesis was to identify genetic markers associated with paclitaxel-induced peripheral neuropathy. First, we evaluated the role of previously proposed genetic markers. And second, we performed whole exome and targeted next generation sequencing to discover new genetic variants associated with the neuropathy.

With regard to the previously proposed genetic variants, we confirmed the association of the common polymorphisms rs7349683 in *EPHA5*, rs301927 in *EPHA6* and rs209709 in *EPHA8* with an increased risk of peripheral neuropathy. Concerning *CYP2C8*3*, we only found a tendency towards increased risk.

Applying whole exome sequencing to patients with severe neuropathy, we identified *CYP3A4* defective variants associated with increased risk of severe neuropathy and higher probability of treatment modifications due to this adverse effect. Furthermore, targeted sequencing of candidate genes led us to discover that low frequency coding variants in *EPHA5*, *EPHA6* and *EPHA8* contributed to the susceptibility to paclitaxel-induced neuropathy.

To sum up, this Thesis supports a role for *CYP3A4* and *EPHAs* genetic variants in paclitaxel-induced neuropathy development. It also recognizes the use of next generation sequencing techniques as novel approaches to detect low-frequency variants associated with drug adverse events. Moreover, the involvement of EphA receptors in neuronal repair function suggests they could also be risk markers for other neurotoxic drugs. The genetic markers identified in this Thesis may help to personalize paclitaxel treatment by identifying, beforehand, those patients with an increased neuropathy risk and that may benefit from alternative drugs and an extensive follow-up along the therapy.

RESUMEN

El paclitaxel, un fármaco antimitótico ampliamente utilizado en el tratamiento de tumores sólidos, tiene la neuropatía periférica como su toxicidad limitante de dosis. En la mayoría de los pacientes, esta neuropatía desaparece al finalizar el tratamiento; sin embargo, hay casos en los que la neuropatía es tan severa que los nervios quedan irreversiblemente dañados y la calidad de vida del paciente resulta afectada de forma permanente. Además, habitualmente se tiene que reducir la dosis del paclitaxel o suspender el tratamiento debido a la neuropatía, lo que puede resultar en un tratamiento sub-óptimo de la enfermedad y en un incremento del riesgo de recaída. Se ha sugerido que la variación genética puede explicar una parte sustancial de la variabilidad inter-individual observada en la toxicidad del paclitaxel. Por ello, el objetivo principal de esta Tesis fue la identificación de marcadores genéticos asociados a neuropatía periférica producida por el paclitaxel. Primero evaluamos el papel de marcadores genéticos sugeridos previamente. En segundo lugar llevamos a cabo estudios de secuenciación de exoma completo y de secuenciación de genes candidatos para descubrir nuevas variantes genéticas asociadas a la neuropatía.

Con respecto al primer punto, confirmamos la asociación de los polimorfismos rs7349683 en *EPHA5,* rs301927 en *EPHA6* y rs209709 en *EPHA8* con un mayor riesgo de neuropatía periférica. En relación a la variante *CYP2C8*3,* solo detectamos una tendencia a mayor riesgo de neuropatía.

Mediante la secuenciación de exomas completos identificamos variantes que producían la pérdida de función del *CYP3A4* en pacientes con neuropatías severas. En concreto, encontramos que los portadores de estas variantes tenían mayor riesgo de sufrir neuropatía severa y mayor probabilidad de tener modificaciones de tratamiento debidas a este efecto adverso. Además, la secuenciación de genes candidatos nos permitió descubrir que variantes codificantes de baja frecuencia en *EPHA5, EPHA6* y *EPHA8* contribuían a la susceptibilidad de desarrollar neuropatía producida por paclitaxel.

En resumen, esta Tesis respalda la implicación de variantes genéticas en *CYP3A4* y en *EPHAs* en el desarrollo de la neuropatía inducida por el paclitaxel. Además, subraya la utilidad de las técnicas de secuenciación de nueva generación como estrategias idóneas para detectar variantes de baja frecuencia asociadas a efectos adversos producidos por fármacos. La función de los receptores de efrina tipo A en la reparación neuronal sugiere que éstos también podrían ser marcadores de riesgo para otros compuestos neurotóxicos. Los marcadores genéticos identificados en esta Tesis podrían ayudar a la personalización del tratamiento con paclitaxel, identificando de antemano, pacientes con alto riesgo de sufrir neuropatía y que podrían beneficiarse de fármacos alternativos y de un seguimiento más estrecho durante la terapia.

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ABBREVIATIONS

ADR: Adverse Drug Reaction

ABCB1: ABC transporter B family member 1 or P-glycoprotein

ADL: Activities of Daily Life

AIDS: Acquired Immune Deficiency Syndrome

ARHGEF10: Rho Guanine Nucleotide Exchange Factor 10

CI: Confidence Interval

CMT: Charcot-Marie-Tooth disease

CIPN: Chemotherapy-Induced Peripheral Neuropathy

CNV: Copy Number Variation

CYP2C8: Cytochrome P450 2C8

CYP3A4: Cytochrome P450 3A4

CYP3A5: Cytochrome P450 3A5

DHPLC: Denaturing High Performance Liquid Chromatography

DHTKD1: DeHydrogenase E1 and TransKetolase Domain containing protein 1

DNA: DeoxyriboNucleic Acid

EPHA or EphA: EPHrin receptor tyrosine kinases, subclass A

EPHA4: EPHrin type-A receptor 4

EPHA5: EPHrin type-A receptor 5

EPHA6: EPHrin type-A receptor 6

EPHA8: EPHrin type-A receptor 8

EMA: European Medicines Agency

FDA: Food and Drug Administration

FGD4: FGD1-related F-actin binding protein

FZD3: Frizzled-3

G-CSF: Granulocyte-Colony Stimulating Factor

GSK3β: Glycogen Synthase Kinase-3β

GWAS: Genome-Wide Association Study

HEK293: Human Embryonic Kidney 293

HR: Hazard Ratio

IKBKAP: IKappaB Kinase complex-Associated Protein

INDEL: INsertion/DELetion

LD: Linkage Disequilibrium

LLN: Lower Limit of Normal

LOF: Loss-Of-Function

MAP: Microtubule-Associated Protein

MAPT: Microtubule-Associated Protein Tau

mRNA: messenger RiboNucleic Acid

NCI-CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events

NGS: Next Generation Sequencing

OATP1B1: Solute carrier Organic Anion Transporter family member 1B1

OATP1B3: Solute carrier Organic Anion Transporter family member 1B3

OR: Odds Ratio

PCR: Polymerase Chain Reaction

PIK3IP1: Phosphoinositide-3-Kinase Interacting Protein 1

PRX: PeRiaXin

QST: Quantitative Sensory Test

RWDD3: RWD domain-containing protein 3

SEPT9: SEPTin 9

SGCG: Gamma-SarcoGlyCan

SH3TC2: SH3 Domain and TetratriCopeptide repeat-containing protein 2

SKAT: Sequence Kernel Association Test

SLCO1B1: SoLute Carrier Organic anion transporter family member 1B1

SLCO1B3: SoLute Carrier Organic anion transporter family member 1B3

SNP: Single Nucleotide Polymorphism

STR: Short Tandem Repeat

TECTA: Alpha-TECTorin

TNS: Total Neuropathy Score

TUBB2A: TUBulin Beta-2A chain

ULN: Upper Limit of Normal

VNTR: Variable Number Tandem Repeats

WES: Whole Exome Sequencing

WGS: Whole Genome Sequencing

XKR4: XK-Related protein 4

INTRODUCTION

1. Cancer pharmacogenetics

Individuals exhibit a great heterogeneity in the way they respond to clinical drugs (Lin, 2007, Evans et al., 2001). Standard drug therapy may result in life-threatening adverse drug reactions (ADR) in some patients, while others may lack the desired therapeutic effect. This variability represents a major challenge in the clinics. It has been estimated that the overall incidence of severe ADR in hospitalized patients is around 6%, making ADR between the fourth and sixth leading cause of death in developed countries (Lazarou et al., 1998, Pirmohamed and Park, 2001). This is of special relevance for drugs with narrow therapeutic indexes such as anti-cancer agents in which the dose required to obtain a therapeutic effect is close to the one that produces toxicity, and where treatments are in general very toxic. Regarding drug response, the efficacy rates observed for many widely used therapeutic agents are low, ranging from 25% to 80% (Spear et al., 2001).

Many factors have been shown to contribute to the variability in drug responses: 1) environmental (e.g. concomitantly administered drugs, nutritional factors, alcohol consumption); 2) pathological (e.g. concomitant diseases); 3) physiological (e.g. age, gender, body mass index); 4) clinical (e.g. mis-dosing, medication errors) and 5) genetic factors (e.g. genetic variants in drug metabolizing enzymes critically altering drug pharmacokinetics) (Weinshilboum and Wang, 2004).

The recognition that a part of this variation is inherited initiated the field of pharmacogenetics fifty years ago (Meyer, 2004). Pharmacogenetics studies how genetics affects drug response. It has been described that any two humans differ only in 0.1% of their genome (Kruglyak and Nickerson, 2001) and this is mainly due to polymorphisms. A polymorphism is defined as a variant with a minor allele frequency >1%, when the frequency is <1%, the variant is classified as low frequency/ rare. Single nucleotide polymorphisms (SNP) are the most common genetic variation with more than 15 million SNPs distributed throughout the human genome. When located in a gene, they can change the encoded amino acids (non-synonymous variant) or can be silent (synonymous variant) or simply occur in noncoding regions. They may influence promoter activity, messenger RNA (mRNA) stability or splicing efficiency among others processes (Komar, 2009). There are other forms of genetic variation including small insertion and deletions (INDELs), short tandem repeats (STR) also known as microsatellites, variable number tandem repeats (VNTR), and variants spanning large segments of DNA (>1kb) known as copy number variations (CNVs) (Haraksingh and Snyder, 2013). Several international projects have been developed to compile genetic variation among different populations (e.g. HapMap, the 1000 Genomes Project) and among different cancer types (e.g. The Cancer Genome Atlas, the International Cancer Genome Consortium) leading to the creation of multiple databases (e.g. dbSNP, Ensembl, UCSC browser, COSMIC, cBioPortal) (Apellaniz-Ruiz et al., 2016).

Regarding the oncology field, despite the significant advances made during the last decades, due to the aggressiveness of these treatments, anti-cancer drugs still cause a high number of ADRs. Some of these lead to treatment discontinuation while others can be fatal. In addition, therapy failure is still high. Indeed, cancer treatment is especially complex because a combination of inherited variations within the individual (germline) and acquired variations within the tumor (somatic) influence disease outcome and the response/ toxicity to the drug therapy (Paugh et al., 2011, Filipski et al., 2014). Germline variation may alter drug pharmacokinetics and pharmacodynamics leading to toxicity and/or lack of efficacy. One example in pharmacokinetics is the use of genetic testing to identify patients with thiopurine-S-methyltransferase deficiency to avoid the development of fatal hematopoietic toxicity when treated with mercaptopurine (Evans and Johnson, 2001, Patel and Papachristos, 2015). On the other hand, somatic mutations can be relevant to predict tumor response (Paugh et al., 2011). This is the case of Vemurafenib, a selective B-Raf inhibitor that has shown extraordinarily results in metastatic melanoma patients with V600E mutation in *BRAF* (Hertz and McLeod, 2013).

Therefore, if differences in drug outcomes are linked to genetic variants, it could be possible to perform a genetic test to select the best medication and dose for a patient and personalize cancer therapy (Paugh et al., 2011, Patel et al., 2014). Advances in cancer pharmacogenomics, in large part derived from the development and implementation of genotyping panels and next generation sequencing (NGS) technologies, are providing a more complete view of the human genome and improving cancer treatment (Gillis et al., 2014, McCarthy et al., 2013).

2. Taxanes

Microtubules, crucial components of the cytoskeleton, are highly dynamic polymers composed of α-tubulin and β-tubulin heterodimers. They play key roles in cell movement, intracellular transport and cell division, assuring the integrity of the segregated DNA. For decades they have been known as effective targets for cancer therapy (Desai and Mitchison, 1997) and nowadays different microtubule binding anti-cancer agents (e.g. taxanes, vinca alkaloids, epothilones) are used as chemotherapeutic drugs for the treatment of several solid and hematologic tumors (Jordan and Wilson, 2004, Perez, 2009, Pasquier and Kavallaris, 2008).

Taxanes are among the most important additions to the chemotherapeutic arsenal in the late twentieth century. The most widely used taxanes are paclitaxel (Taxol®, Bristol-Meyers Squibb) and the semi-synthetic analog docetaxel (Taxotere®, Sanofi).

The need for new compounds with higher solubility and less toxicity have guided the development of new taxanes such as the already approved nanoparticle albumin–bound paclitaxel (nab-paclitaxel, Abraxane®, Celgene) and cabazitaxel (Jevtana®, Sanofi), or others that have entered clinical trials (e.g. Tesetaxel®, Opaxio®, Larotaxel®, Taxopresin® (Yared and Tkaczuk, 2012, Muggia and Kudlowitz, 2014)) (Figure 1).

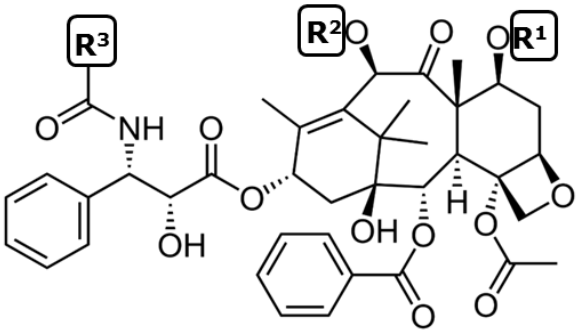

Figure 1. Structure of paclitaxel, docetaxel and cabazitaxel. Residues in black boxes differ between paclitaxel (**R**^{1}=H, **R**^{2}=Ac, **R**^{3}=Ph), docetaxel (**R**^{1}=**R**^{2}=H, **R**^{3}=O-t-Bu) and cabazitaxel (**R**^{1}=**R**^{2}=CH3, **R**^{3}=O-t-Bu).

These cytotoxic drugs bind with high affinity to the β-tubulin subunit on the inside surface of the microtubule leading to microtubule stabilization (Figure 2). This suppresses the microtubule dynamics required for accurate chromosome segregation, and results in mitotic arrest and finally, cell death through apoptosis (Rowinsky, 1997, Fauzee, 2011). In addition to the antimitotic role, taxanes have been shown to exert antiangiogenic effects (Wang et al., 2003, Guo et al., 2003) although the exact mechanisms are not well understood. Either as single agents or in combination with other chemotherapeutic or targeted agents, taxanes have demonstrated significant activity against many solid tumors and are among the standard first-line chemotherapeutic treatments (e.g. breast, ovarian, lung, pancreatic and prostate cancer).

3. Paclitaxel

Paclitaxel, a natural alkaloid derived from the bark of the *Taxus Brevifolia* (Pacific yew conifers), was the first taxane to be isolated and used in the clinic (Wani et al., 1971). It binds to a specific pocket in the β-tubulin subunit formed predominantly by hydrophobic residues. This binding induces a conformational change in the M-loop that leads to increased lateral interactions with H1-S2 loops of neighboring β-tubulin subunits (Nogales et al., 1998, Mitra and Sept, 2008) (Figure 2). This results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions, and thus preventing proliferation of tumor cells (Schiff et al., 1979, Schiff and Horwitz, 1980, Jordan and Wilson, 2004).

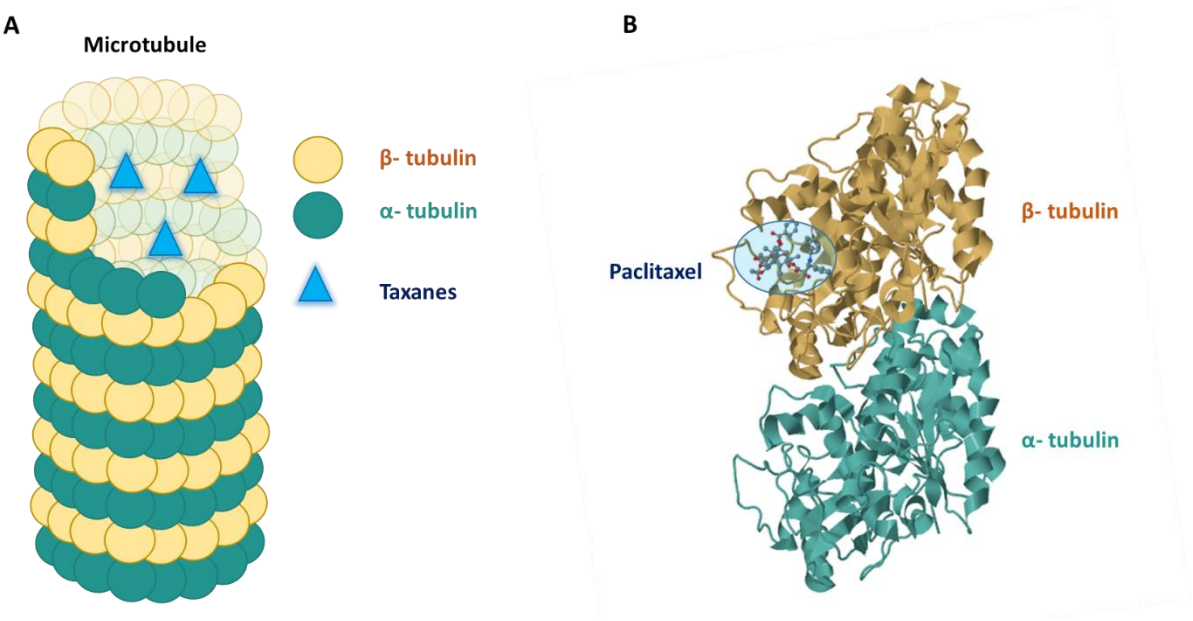

Figure 2. Taxanes binding site. A) Microtubules are made of α-β-tubulin dimers and taxanes bind to β-tubulin subunit on the inside surface of the microtubule. B) Paclitaxel binding site.

The clinical use of this microtubule-binding drug is approved by the US Food and Drug Administration (FDA) and The European Medicines Agency (EMA) for the treatment of ovarian (McGuire et al., 1996), breast (Holmes et al., 1991), non-small-cell lung cancer (Murphy et al., 1993) and AIDS-related Kaposi's sarcoma (Fauzee, 2011), while nab-paclitaxel is approved for lung (Socinski et al., 2012), breast (Gradishar et al., 2005) and pancreatic cancer (Von Hoff et al., 2011). Moreover, various studies have also reported antitumor activity against other tumors (Rowinsky 1997).

Paclitaxel is administered intravenously and is largely distributed to all tissues except the testes and the central nervous system. Hepatic metabolism followed by biliary excretion is the major pathway of paclitaxel elimination (Rowinsky, 1997). The OATP1B3 and OATP1B1 transporters are in charge of introducing paclitaxel into the hepatocytes (Smith et al., 2005, Gui et al., 2008, Nieuweboer et al., 2014, Svoboda et al., 2011, Marada et al., 2015) and there, it is metabolized by different cytochromes P450 (CYP2C8, CYP3A4 and CYP3A5) into 6α-hydroxypaclitaxel, 3'-p-hydroxypaclitaxel and 6α, 3'-p-dihydroxypaclitaxel (Harris et al., 1994, Rahman et al., 1994, Sonnichsen et al., 1995). ABCB1, also known as P-glycoprotein, is in charge of the efflux transport into the bile canaliculi (Jang et al., 2001).

3.1. Clinical problems associated with paclitaxel treatment

Paclitaxel has been widely used since its approval in the 1990s as an effective antineoplasic agent. Unfortunately, intrinsic or acquired drug resistance and adverse effects are relevant clinical problems.

3.1.1. Paclitaxel resistance

Many resistance mechanisms have been described including the up-regulation of the drug efflux transporter P-glycoprotein (Gottesman et al., 2002, Orr et al., 2003, Horwitz et al., 1993), alterations in the expression of β-tubulin isotypes (Kavallaris et al., 1997, Mozzetti et al., 2005, Sève and Dumontet, 2008) or in the microtubule assembly regulatory proteins (Larsson et al., 1999, Balachandran et al., 2003, Zhang et al., 1998, Zhang et al., 1999), aberrant signal transduction pathways (Montgomery et al., 2000, Yu et al., 1998), and changes in proteins such as Bcl-2 (Gazitt et al., 1998, Panvichian et al., 1998), survivin (Zhou et al., 2004), TLR4 (Rajput et al., 2013), prohibitin1 (Patel et al., 2010) or BubR1 (Hu et al., 2013). In addition, altered expression of microRNAs has also been shown to contribute to paclitaxel resistance (Kim et al., 2014, Cui et al., 2013).

3.1.2. Paclitaxel toxicities

Many adverse events have been associated with paclitaxel including bradycardia, hypotension, mucositis, vomiting, edema or alopecia among others (Rowinsky et al., 1993, Suffness, 1995). Nevertheless, the most critical toxicities are:

a) Hypersensitivity reactions: Paclitaxel, due to its poor solubility in water, is marketed commercially in a formulation that contains a solvent system of Cremophor and dehydrated ethanol. Cremophor has been associated with severe, sometimes fatal, hypersensitivity reactions. However, premedication of cancer patients with steroids and antihistamines has greatly reduced the risk of such reactions (Gelderblom et al., 2001, Kloover et al., 2004, Weiss et al., 1990).

b) Myelosuppression: Hematological toxicity is a common adverse event for many cytotoxic drugs. This toxicity derives from their effect on any highly proliferative cells, either tumor cells or normal host cells (e.g. bone marrow cells). Neutropenia is the most frequent hematological toxicity caused by paclitaxel (Rowinsky et al., 1993). It is dose and schedule dependent but not cumulative, suggesting that paclitaxel does not irreversibly affect immature hematopoietic cells (Eisenhauer et al., 1994, Perez, 1998). The incidence of neutropenia increases when prior myelotoxic therapy is used or when paclitaxel is given in combination schedules (Rowinsky et al., 1993, Albain et al., 2008). Fortunately, the implementation of granulocyte-colony stimulating factors (G-CSF) and the decrease of paclitaxel infusion times (e.g. from 24h to 3h), have diminished the clinical complications of this side effect.

c) Neuropathy: This is the current paclitaxel dose-limiting toxicity (Rowinsky et al., 1993). The incidence and severity of the neuropathic manifestations are related to the drug schedule and the cumulative dose (Postma et al., 1995, Argyriou et al., 2008). Paclitaxel affects predominantly large sensory nerves causing sensory symptoms although motor alterations can also be observed (Mielke et al., 2006, Scripture et al., 2006). Paclitaxel dose reductions and treatment discontinuations as a consequence of the neuropathy are common, and can compromise treatment success. Regarding the duration of the symptoms, in most patients they disappear months after finishing the treatment, but in severe cases the nerve damage can be irreversible affecting permanently the patients' quality of life (Lipton et al., 1989). In addition, the lack of an effective preventive or symptomatic treatment (Wolf et al., 2008) makes neuropathy the most relevant toxicity of paclitaxel.

4. Peripheral neuropathy

The peripheral nervous system is a network of nerves that connect the brain and the spinal cord (the central nervous system) to the entire human body. These nerves control sensation, movement, motor coordination and involuntary functions. Peripheral neuropathy develops as a result of damage to these nerves. The incidence of peripheral neuropathy in the population has been estimated between 2% and 8% (Martyn and Hughes, 1997). The damage or dysfunction can affect the neuron cell body "neuronopathy", the axons "axonopathy", the myelin sheath "myelinopathy" or the neuromuscular junctions. Neuropathy can involve one nerve (mononeuropathy), two or more nerves in separate areas (multiple mononeuropathy) or many peripheral nerves in a generalized and homogenous manner (polyneuropathy), and it can affect different types of nerves (e.g. sensory or motor) (England and Asbury, 2004, Chamberlin and Narins, 2005). Peripheral neuropathy can be divided into: a) hereditary neuropathy, when it is caused by genetic defects (e.g. Charcot-Marie-Tooth disease); b) acquired neuropathy, when it is caused by environmental factors such as toxins, drugs, trauma, illness, dysmetabolic states or infection (e.g. diabetes, alcoholism, chemotherapy, AIDS) and c) idiopathic neuropathy, when the causes are not known (Barohn and Amato, 2013, Martyn and Hughes, 1997).

4.1. Hereditary peripheral neuropathy

Charcot-Marie-Tooth (CMT) diseases comprise a genetically heterogeneous group of disorders. The majority of CMT are transmitted in a monogenic (dominant, recessive, or X-linked), highly penetrant mode and are one of the most common inherited disorders in humans, with an estimated prevalence of one case per 2,500 individuals (Skre, 1974).

CMT is characterized by a progressive and length-dependent degeneration of the peripheral nerves resulting in muscle weakness and wasting in distal limbs, feet and hands, sensory loss, decreased reflexes and foot deformities (Rossor et al., 2015, Klein et al., 2013). Approximately 80 genes representing key elements in biological mechanisms have been reported to cause CMT (Timmerman et al., 2014).

4.2. Chemotherapy-induced peripheral neuropathy

Chemotherapy-induced peripheral neuropathy (CIPN) is a type of acquired neuropathy, and is a major side effect of many commonly used chemotherapeutic agents (e.g. platinum drugs, taxanes, epothilones, vinca alkaloids, thalidomide, bortezomib) (Quasthoff and Hartung, 2002, Gutiérrez-Gutiérrez et al., 2010, Miltenburg and Boogerd, 2014). It is estimated to affect a high percentage of cancer treated patients (ranging from 12 to 96% depending on the drug), some of which will have dose reductions or treatment discontinuation, which can compromise drug efficacy (Speck et al., 2013, Bhatnagar et al., 2014). In addition, CIPN can lead to permanent symptoms and disabilities (Seretny et al., 2014) negatively altering daily life activities, in some cases greatly compromising the quality of life of the survivor (Ezendam et al., 2014). Nowadays with millions of cancer survivors worldwide, it is critical to prevent and to have effective treatments against CIPN.

4.2.1. Chemotherapy-induced peripheral neuropathy symptoms and diagnosis

In patients with CIPN, sensory nerves are commonly affected leading to paresthesia (abnormal sensation such as tingling, pricking, itching or burning), dysesthesia (unpleasant abnormal sensation such as hyperalgesia or allodynia), numbness and pain. The early symptoms usually affect the tips of the toes and/or fingers and progress with a "glove and stocking" distribution (Figure 3). Sensory loss in feet and legs can cause sensory ataxia and gait disorders. Moreover, motor and autonomic symptoms can also happen (Park et al., 2013b, Quasthoff and Hartung, 2002, Grisold et al., 2012).

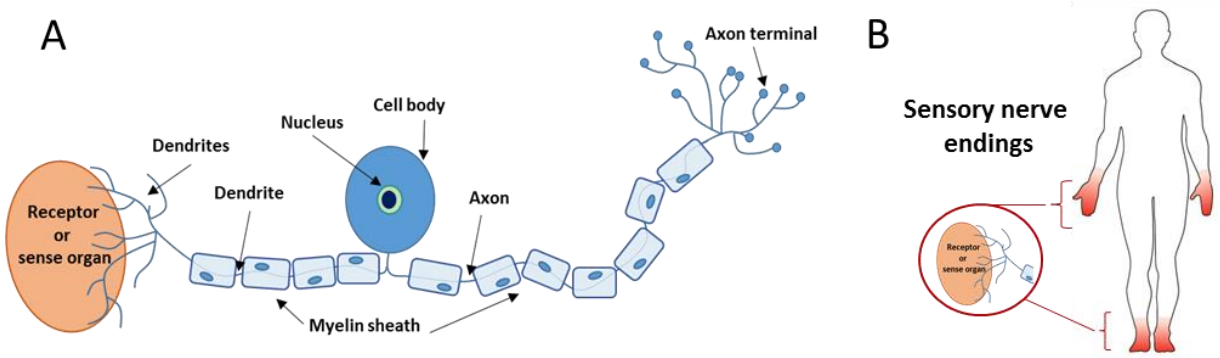

Figure 3. Sensory neurons and peripheral neuropathy. A) Representation of the parts of a sensory neuron. **B)** Glove and stocking distribution of peripheral neuropathy.

There is no gold standard to assess CIPN and therefore, the studies on CIPN are based on descriptions of this toxicity in medical records, clinical examinations of the patients or patient-reported outcomes. Neurologists usually evaluate sensory perception (touch, vibration, and proprioception), coordination and deep tendon reflexes. Instrumental examinations including electromyography, nerve conduction studies and nerve biopsies are less frequently used in the diagnosis due to the limited usefulness in detecting early signs and the discomfort produced in patients (Cavaletti et al., 2010, Park et al., 2013b).

Several grading scales are utilized to evaluate CIPN severity. However, when the scales include subjective items, they often lead to inter-observer inconsistencies (Postma et al., 1998) or to under-estimation and under-reporting of the neuropathy grade compared to patient self-reports (Fromme et al., 2004, Basch et al., 2006). Owing to these issues, scales based on clinical and instrumental examination, and patients' questionnaires have been developed to improve the accuracy, reliability and effectiveness of neuropathy assessment.

The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) (Trotti et al., 2003) and the Total Neuropathy Score (TNS) (Cornblath et al., 1999, Cavaletti et al., 2003) are among the most common clinical grading scales used (Table 1). Other scales such as the World Health Organization Scale (Miller et al., 1981), Eastern Cooperative Oncology Group Scale (Oken et al., 1982), Ajani Scale (Ajani et al., 1990) are also used. Moreover, drug specific scales as the Functional Assessment of Cancer Therapy-Taxane (Cella et al., 2003) or the Oxaliplatin-associated neuropathy questionnaire (Leonard et al., 2005) have been developed.

In cancer patients, the most frequently used scale is the NCI-CTCAE, which includes neurosensory and neuromotor parameters. It is fast and easy to perform, however, it results in moderate inter-observer disagreement (45 to 81% of agreement between clinicians), although training the observers has been demonstrated to increase accuracy (Postma et al., 1998, Cavaletti et al., 2013). Regarding TNS, it combines symptom scores with objective measurements of sensory loss and neurophysiological parameters and it has been shown to be more sensitive and precise to detect neuropathy changes during chemotherapy than the NCI-CTCAE scale (Cavaletti et al., 2006, Cavaletti et al., 2007, Frigeni et al., 2011, Chaudhry et al., 2008). The development of faster and simpler versions of the TNS, such as the TNS clinical, based on clinical items (e.g. tendon reflexes, vibration perception) or the TNS reduced, without quantitative sensory testing, provides with additional resources for CIPN evaluation (Cavaletti et al., 2003, Cavaletti et al., 2007). Despite the accuracy of the TNS, it is commonly perceived as complicated to perform in a routinely cancer consultation because it requires specific instrumentation to perform the clinical examination and because it is time consuming.

At any rate, it is relevant to highlight that many studies assessing the presence and severity of CIPN with different scales have showed a good correlation between TNS and NCI-CTCAE (Cavaletti et al., 2003, Cavaletti et al., 2006, Cavaletti et al., 2013).

Recently, patient-reported toxicities have shown to detect higher neuropathy grades and at an earlier onset than those reported by clinicians (Sasane et al., 2010, Cirillo et al., 2009, Di Maio et al., 2016). This strongly supports the incorporation of patient-reported outcomes into toxicity reporting. Among the patient self-reported questionnaires, we can find the European Organization for Research and Treatment of Cancer QTQ-CIPN20 questionnaire (Postma et al., 2005), the Functional Assessment of Cancer/Gynecologic Oncology Group-Neurotoxicity questionnaire (Calhoun et al., 2003) and the Patient Neurotoxicity Questionnaire (Hausheer et al., 2006).

Numerous initiatives are underway to identify the best approach to measure CIPN. Meanwhile, available data suggests that appropriate, standardized, and objective assessment tools joined with patient-reported symptoms would be needed to accurately assess CIPN.

4.2.2. Chemotherapy-induced peripheral neuropathy prevention and treatment

Another relevant factor regarding CIPN is the lack of neuroprotective and effective treatment despite the many trials performed with different drugs. Nowadays no agent can prevent CIPN and only few drugs are recommended to decrease pain and sensory symptoms (e.g. duloxetine or tricyclic antidepressants) (Hershman et al., 2014, Piccolo and Kolesar, 2014). Thus, the most successful approaches to prevent CIPN are dose modifications or treatment interruption. The understanding of the mechanisms by which chemotherapy drugs cause neuropathy will allow to developing neuroprotective strategies and valid treatments.

Table 1. Most common neuropathy grading scales used in the clinics

Scale	Parameter	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Common Terminology Criteria for Adverse Events (CTCAE) v4.0	Motor symptoms	None	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self-care ADL; assistive device indicated	Life-threatening consequences; urgent intervention indicated
	Sensory symptoms	None	Asymptomatic; loss of deep tendon reflexes or paresthesia	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self-care ADL	Life-threatening consequences; urgent intervention indicated
Scale	**Parameter**	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**
Total Neuropathy Score (TNS)*	Sensory symptoms	None	Symptoms limited to finger or toes	Symptoms extend to ankle or wrist	Symptoms extend to knee or elbow	Symptoms above knees or elbows, or functionally disabling
	Motor symptoms	None	Slight difficulty	Moderate difficulty	Requires help/assistance	Paralysis
	Autonomics symptoms	0	1	2	3	4 o 5
	Pin sensibility	Normal	Reduced in finger/toes	Reduced up to wrist/ankle	Reduced up to elbow/knee	Reduced above elbow/knee
	Vibration sensibility	Normal	Reduced in finger/toes	Reduced up to wrist/ankle	Reduced up to elbow/knee	Reduced above elbow/knee
	Strength	Normal	Mild weakness	Moderate weakness	Severe weakness	Paralysis
	Tendon reflex	Normal	Ankle reflex reduced	Ankle reflex absent	Ankle reflex absent, others reduced	All reflexes absent
	Vibration sensation (QST vibration)	Normal to 125% of ULN	126-150% of ULN	151-200% of ULN	201-300% of ULN	>300% of ULN
	Sural amplitude	Normal/Reduced to <5% of LLN	76-95% of LLN	51-75% of LLN	26-50% of LLN	0-25% of LLN
	Peroneal amplitude	Normal/Reduced to <5% of LLN	76-95% of LLN	51-75% of LLN	26-50% of LLN	0-25% of LLN

In CTCAE, grade 5 is death. ADL= Activities of daily life; QST= Quantitative Sensory Test; ULN= Upper Limit of Normal; LLN= Lower Limit of Normal. Activities of daily living are basic tasks of everyday life (i.e. walking, eating, bathing) whereas instrumental activities of daily living capture a wide range of more complex activities (i.e. cooking, driving, shopping).

* TNS has a total of 10 items each rated on a scale of 0-4, where 0 is normal and 4 is most severe, yielding a total possible score of 0-40. A higher total score indicates a more severe neuropathy.

5. Paclitaxel-induced peripheral neuropathy

In the specific case of paclitaxel, some patients treated with this drug will present a long-lasting peripheral neuropathy. The incidence of peripheral neuropathy among patients treated with paclitaxel can be up to 70%, depending on the dose and schedule, with high grades occurring in up to 30% of patients (Rivera and Cianfrocca, 2015, Schneider et al., 2012, Pace et al., 2007). Peripheral neuropathy improves in most patients within 3-6 months after cessation of the treatment, but symptoms and disability can be permanent in a significant percentage of the patients (Tanabe et al., 2013, Scripture et al., 2006, Pignata et al., 2006).

5.1. Paclitaxel-induced peripheral neuropathy pathophysiology

Paclitaxel-induced peripheral neuropathy has been described as a symmetrical and distal sensory neuropathy with less prominent motor involvement. Reduction in motor skills and walking ability may occur in severe cases. Sensory symptoms include numbness, tingling and pain. There is also loss of proprioception, vibration, temperature and tendon reflexes (Argyriou et al., 2008). This phenomenon begins at the distal nerve endings of the longest nerves and then spreads centrally (Cavaletti et al., 1995, Cavaletti et al., 1997). The exact mechanism that underlies paclitaxel-induced neuropathy is not known although the inhibition of microtubule dynamics disrupting axonal transport is widely accepted (Gornstein and Schwarz, 2014, Cavaletti et al., 1995, Cavaletti et al., 1997, Peters et al., 2007). Increased axonal microtubule stability or polar reconfiguration (Shemesh and Spira, 2010) following paclitaxel treatment may alter axonal transport resulting in abnormal nerve physiology (Nakata and Yorifuji, 1999), and altered mitochondrial supply (Park et al., 2013a, Canta et al., 2015), leading to a loss of axonal integrity or axonal degeneration. The reason why paclitaxel produces preferentially impairment of sensory neurons over motor ones remains unclear (Gornstein and Schwarz, 2014). The morphological changes observed in neurons are axonopathy, severe nerve fiber loss, axonal atrophy and secondary demyelination with little axonal regeneration (Quasthoff and Hartung, 2002, Dougherty et al., 2004).

An acute pain syndrome has also been found in up to 70% of paclitaxel-treated patients (Loprinzi et al., 2011). It is characterized by myalgia or arthralgia that usually develops within 1 to 3 days of drug infusion and resolves in a week (Rowinsky et al., 1993, Saibil et al., 2010, Tofthagen et al., 2013). It is frequently observed with short and high-dose infusions (Moulder et al., 2010, Loprinzi et al., 2011) and has been reported to be predictive of sensory neuropathy (Reeves et al., 2012). The mechanism underlying this syndrome has been related with a sensitization of nociceptors and their fibers by pro-inflammatory cytokines (IL-6, IL-8, IL1β, TNF-α) (Loprinzi et al., 2007, Wang et al., 2012).

5.2. Paclitaxel-induced peripheral neuropathy risk factors

Not all patients receiving paclitaxel will develop peripheral neuropathy, and among affected patients there is high inter-individual difference in the severity of the toxicity. There are many factors that can influence neuropathy risk and severity.

Regarding treatment type, paclitaxel schedule and cumulative dose have been identified as risk factors. Patients with higher doses per cycle (≥250 mg/m^2) and higher cumulative doses are at greater risk of neuropathy (Postma et al., 1995, Rowinsky et al., 1993). Drug infusion-time has also been associated with neuropathy incidence. In this case, shorter infusion times are associated with higher neuropathy risk (Moulder et al., 2010, Smith et al., 1999). Concerning the combination of paclitaxel with different drugs, the co-administration of paclitaxel with other neurotoxic drugs such as cisplatin has consistently been demonstrated to increase the development of neuropathy (Chaudhry et al., 1994, Kudlowitz and Muggia, 2013). It is also important to take into account that co-administered drugs inhibiting or inducing liver drug metabolizing enzymes may alter paclitaxel metabolism and alter the risk to suffer from neuropathy (Badyal and Dadhich, 2001).

Patient predisposing factors such as higher age (Tanabe et al., 2013, Chen et al., 2006, Akerley et al., 2003), hyperglycemia (Akerley et al., 2003) and non-European origin (Hertz et al., 2013) have been associated with increased risk of neuropathy. Other conditions such as alcohol intake, diabetes, liver disease or previous neuropathy diseases (e.g. Charcot-Marie-Tooth) have been described to predispose to paclitaxel-induced neuropathy (Weimer and Podwall, 2006, Rowinsky et al., 1993, Lee and Swain, 2006, Chopra and Tiwari, 2012, de la Morena Barrio et al., 2015). Moreover, there are increasing amounts of evidence supporting that the patient genetic background can influence drug response. In a pharmacogenomic study analyzing 29 commonly prescribed cytotoxic drugs *in vitro*, estimated that a high proportion of the variability observed in paclitaxel response was inherited (Peters et al., 2011).

6. Genetic variants associated with paclitaxel-induced peripheral neuropathy

Genetic polymorphisms are a major source of individual differences in drug response. Therefore, the identification of genetic markers of paclitaxel-induced peripheral neuropathy could lead to an individualized neuropathy risk assessment and personalization of the treatment. As a result, many efforts have been done to accomplish this goal.

6.1. Genotyping-based strategies

Genotyping is performed to determine the genetic makeup of an individual (genotype), by examining the DNA sequence at a specific position and comparing it to a reference DNA sequence (Shi et al., 1999).

Genotyping methods can be carried out to evaluate a single variant (e.g. through gel electrophoresis-based or fluorescent dye-based techniques) or they can be applied simultaneously to hundreds of thousands of variants (e.g. through high-throughput technologies based on high-density oligonucleotide SNP arrays).

6.1.1. Genotyping-based candidate gene approaches

Increased susceptibility to neuropathy can be caused by an alteration in the drug pharmacokinetic (drug absorption, distribution, metabolism and excretion) and/ or pharmacodynamic pathways (drug targets and mechanisms of drug action) (Pirmohamed and Park, 2001). Thus, several studies have studied common polymorphisms in genes encoding paclitaxel metabolizing enzymes (*CYP2C8, CYP3A4* and *CYP3A5*), paclitaxel transporters (*SLCO1B3, SLCO1B1* and *ABCB1*) and paclitaxel targets (tubulins and microtubule-associated proteins) in relation with the drug neuropathy.

CYP2C8

CYP2C8 is the major enzyme metabolizing paclitaxel to 6α-hydroxypaclitaxel, a process that occurs in the liver (Rahman et al., 1994, Sonnichsen et al., 1995). Coding polymorphisms in *CYP2C8* include *CYP2C8*2, CYP2C8*3* (rs11572080 plus rs10509681), *CYP2C8*4* and *CYP2C8*6* alleles, among others. *CYP2C8*3* allele, with a frequency of 12% in the Caucasian population, encodes for a protein with Arg139Lys and Lys399Arg substitutions. Several *in vitro* studies have found that *CYP2C8*3* exhibits an altered activity in the metabolism of several drugs (Dai et al., 2001, Soyama et al., 2001, Rowbotham et al., 2010, Bahadur et al., 2002), including paclitaxel (Dai et al., 2001). In addition, Gréen *et al* and Bergmann *et al* found that patients heterozygous for *CYP2C8*3* had lower clearance of paclitaxel compared to patients homozygous for *CYP2C8*1* (wild type) (Gréen et al., 2009, Bergmann et al., 2011a), although another pharmacokinetics study did not detect an effect (Henningsson et al., 2005). Leskela *et al* and Hertz *et al* found that *CYP2C8*3* allele was associated with a high risk of neuropathy by analyzing the accumulated paclitaxel dose causing a grade 2 or higher neuropathy (HR=1.72, P=0.032; n=118 patients) and (HR=1.93, P=0.006, n=209 Europeans, and P=0.043, n=107 African-Americans) (Leskelä et al., 2011, Hertz et al., 2013). The association was replicated in two independent studies (n=412, HR=1.72 (Hertz et al., 2014) and n=119, OR=1.49 (Boora et al., 2016)). However, other groups did not find an association (Abraham et al., 2014, Marsh et al., 2007, Baldwin et al., 2012, Ofverholm et al., 2010, Bergmann et al., 2011b, Rizzo et al., 2010). Potential factors that could account for these differences include: differences in neuropathy grade assessments, sample size, paclitaxel regimens and ethnicity. At any rate, an in depth characterization of *CYP2C8*3* allele on paclitaxel metabolism and toxicity remains to be performed.

Additional variants with decreased *in vitro* enzyme activity have been described (e.g. *CYP2C8*2* (Dai et al., 2001) and *CYP2C8*4* (Singh et al., 2008)).

One study found an association for *CYP2C8*4* allele (rs1058930, Ile264Met) with neuropathy (HR= 1.38, 95%CI=1.03–1.86, P=0.04 (Abraham et al., 2014)) and this is supported by a *CYP2C8.4* decreased paclitaxel metabolism detected in bacteria models (Singh et al., 2008) and in patients (18% lower mean clearance; (Bergmann et al., 2011a)). However, the association between *CYP2C8*4* and paclitaxel neuropathy has not been replicated in other studies (Hertz et al., 2014, Leskelä et al., 2011, Henningsson et al., 2005, Baldwin et al., 2012, Marsh et al., 2007, Rizzo et al., 2010). CYP2C8 haplotypes B and C with frequencies of 24 and 22% in Caucasians were associated with a significantly increased and reduced paclitaxel metabolism, respectively, in human liver samples (Rodríguez-Antona et al., 2008). Leskela *et al* detected an association between CYP2C8 haplotype C (rs1113129) with neuropathy protection (HR=0.55, 95%CI=0.34–0.89, P=0.014, (Leskelä et al., 2011)) but follow up studies could not confirm this association (Abraham et al., 2014, Baldwin et al., 2012).

CYP3A4

CYP3A4 is another major paclitaxel metabolizing enzyme, and it catalyzes the 3'-p-hydroxylation of the drug (Harris et al., 1994, Sonnichsen et al., 1995). It is encoded by a highly conserved gene. Unlike other cytochrome P450 enzymes, and despite the fact that it is suggested that up to 90% of CYP3A4 activity variability would have a genetic basis, no common coding polymorphisms have been described to date (Ozdemir et al., 2000, Lamba et al., 2002, Rahmioglu et al., 2011). Genetic databases include many coding variants in *CYP3A4* but they all have a low frequency, there are rare premature stop codons producing truncated proteins (e.g. *CYP3A4*6*, *CYP3A4*20* (Westlind-Johnsson et al., 2006) and *CYP3A4*26* (Werk et al., 2014)) and missense variants, some of them conferring reduced CYP3A4 activity (e.g. *CYP3A4*8*).

The most common *CYP3A4* variant alleles proposed to have functional consequences are two noncoding SNPs with an allelic frequency of approximately 5% in the Caucasian population (*CYP3A4*1B* and *CYP3A4*22*). *CYP3A4*1B* is located in CYP3A4 promoter (rs2740574, -392A>G) and carriers of this allele have been associated with lower enzymatic activity compared to those with *CYP3A4*1* (wild type) (Rodríguez-Antona et al., 2005), but other studies did not detect an effect (Lamba et al., 2002, Ball et al., 1999). Concerning paclitaxel-induced neuropathy, only a trend towards higher risk of neuropathy has described for the variant allele in one study (P=0.057 (Leskelä et al., 2011)) (Marsh et al., 2007, Gréen et al., 2008, Bergmann et al., 2012, Ofverholm et al., 2010, Lambrechts et al., 2015, Abraham et al., 2014). *CYP3A4*22* is located in intron 6 (rs35599367, C15389T) and it was first described to affect hepatic CYP3A4 expression and response to statin drugs (Wang et al., 2011).

The variant allele was associated with reduced mRNA levels when compared to wild type allele, accounting for around 10% of the variability observed in CYP3A4 mRNA expression and lower doses of statins and immunosuppressants were suggested for *CYP3A4*22* carriers compared to CYP3A4 wild type individuals (Elens et al., 2011b, Elens et al., 2011c, Elens et al., 2011a). Graan *et al* studied the role of *CYP3A4*22* in paclitaxel-induced neuropathy finding that variant carriers had an increased risk of developing neurotoxicity and validated the findings in an independent series (P=0.043, n=262 and P=0.036, n=239; for combined analysis HR= 22.1, 95%CI=4.7-105, P<0.001) (de Graan et al., 2013).

CYP3A5

CYP3A5 has similar substrate specificity than CYP3A4 (Williams et al., 2002, Zanger and Schwab, 2013). Unlike *CYP3A4*, *CYP3A5* gene is very polymorphic and its activity in Caucasians is determined by *CYP3A5*3* allele (rs776746, G6986A) (Kuehl et al., 2001). *CYP3A5*3* introduces a splicing site in intron 3 resulting in an alternative spliced mRNA that encodes a nonfunctional truncated protein. As a consequence, homozygosity for the *CYP3A5*3* allele results in undetectable enzyme activity whereas the presence of the *CYP3A5*1* allele in an individual is associated with high levels of functional protein. Leskela *et al* found that *CYP3A5*3* was associated with paclitaxel-induced neuropathy protection (OR=0.51, 95%CI=0.30–0.86, P=0.012, n=118, (Leskelä et al., 2011)), but others did not confirm the association (Bergmann et al., 2012, Lambrechts et al., 2015, Marsh et al., 2007, Abraham et al., 2014).

SLCO1B3 and *SLCO1B1*

Regarding the genes encoding for the transporters that introduce paclitaxel into the hepatocytes (i.e. OATP1B3 and OATP1B1), researchers studied missense SNPs with reported functional consequences: rs4149056 (p.V174A) in *SLCO1B1*, and rs4149117 and rs7311358 (p.S112A and p.M233I, respectively) in *SLCO1B3*. However, no significant association for these SNPs has been detected when studying *in vitro* paclitaxel disposition (Smith et al., 2007) nor with paclitaxel-induced neuropathy in patients (Leskelä et al., 2011, Lambrechts et al., 2015). Conversely, the SNP rs3829306 in *SLCO1B1* was significantly associated with neuropathy risk in one study (Leandro-García et al., 2013) but with protection in another (Abraham et al., 2014).

ABCB1

In *ABCB1*, the main protein mediating paclitaxel elimination through efflux transport into the bile canaliculi, three coding variants: rs2032582 (p.A893S), rs1128503 (p.G412G) and rs1045642 (p.I1145I), have been associated with altered drug transport (Leschziner et al., 2007); and a common missense polymorphism (rs9282564, p.N21D) has also been described (Chang et al., 2009).

Concerning paclitaxel pharmacokinetics, Gréen *et al* found that patients heterozygous for rs2032582 variant had a significantly higher clearance of paclitaxel than wild-type patients (Gréen et al., 2009). Henningsson *et al* studied rs1045642 but did not detect any association with paclitaxel pharmacokinetics (Henningsson et al., 2005). Regarding paclitaxel-induced neuropathy, rs2032582 allele has been associated with neuropathy risk in a large series of patients (HR=1.19, P=0.02, n=1303 (Abraham et al., 2014)) and rs1045642 allele with decreased risk of neuropathy (HR=0.71-0.83, (Leskelä et al., 2011, Gréen et al., 2008, Abraham et al., 2014)).

In contrast, other groups have shown that rs1128503, rs2032582 or rs1045642 alleles did not correlate with the occurrence of neurotoxicity in their series of patients (Bergmann et al., 2012, Bergmann et al., 2011b, Ofverholm et al., 2010, Lambrechts et al., 2015, Rizzo et al., 2010, Marsh et al., 2007). Additionally, a polymorphism located in the 5'UTR of *ABCB1* (rs3213619) has been associated with protection against neuropathy (OR=0.12-0.51, (Leskelä et al., 2011, Abraham et al., 2014, Boora et al., 2016)).

Pharmacodynamic genes

In addition, variants in genes encoding paclitaxel target (β-tubulin) and microtubule related proteins have also been studied. Indeed, it has been found that paclitaxel-induced neuropathy has a heritable component driven in part by genes involved in axon outgrowth (Chhibber et al., 2014).

As the cytotoxic effect of paclitaxel requires β-tubulin binding, genetic variants in β-tubulin genes may influence neuropathy susceptibility. All β-tubulins, except for the hematologic-specific β-tubulin VI (Leandro-García et al., 2012a), are highly conserved genes that lack polymorphisms leading to amino acid changes. However, variability in the expression of β-tubulin isotypes due to regulatory polymorphisms has been shown. Our group identified three polymorphisms located at -101 (rs909964), -112 (rs909965), and -157 (rs9501929) in the promoter of β-tubulin IIa gene (*TUBB2A*) that correlated with increased mRNA levels. The rs909964 and rs909965 variants, in total linkage disequilibrium, protected from paclitaxel-induced peripheral neuropathy (HR=0.62, 95%CI=0.42-0.93, P=0.021 in multivariable analysis). This was further supported by the finding that higher *TUBB2A* gene expression correlated with lower levels of paclitaxel-induced apoptosis (P=0.001) in lymphoblastoid cell lines, leading to decreased paclitaxel sensitivity (Leandro-García et al., 2012b). Although Leandro-García *et al* did not find an association for rs9501929 allele, Abraham *et al* did detect it (OR=1.80; 95%CI=1.20–2.72; P=0.005) (Abraham et al., 2014).

Not only β-tubulins but also proteins such as microtubule-associated protein tau (MAPT) (Myers et al., 2007) or tau-related protein glycogen synthase kinase-3β (GSK3β) (Lovestone et al., 1994) are critical for microtubule function.

Given the possible role of microtubule dysfunction in neurotoxicity, Park and colleagues investigated the effects of genetic variation in *MAPT* and *GSK3B* on neurotoxicity in paclitaxel-treated patients. They found a significant association between polymorphism rs6438552 in *GSK3B* and paclitaxel-induced neurotoxicity. The variant allele was associated with reduced neurotoxicity severity ($P \leq 0.05$) and increased tau phosphorylation, which resulted in a reduction of microtubule stabilization which may be beneficial to axonal integrity and thus protecting against neuropathy (Park et al., 2014). Lambrechts *et al* also studied SNPs in genes related to paclitaxel pharmacodynamics (e.g., *MAPT*, tubulins) in 322 patients treated with first-line paclitaxel-carboplatin but no association with neuropathy was found (Lambrechts et al., 2015).

6.1.2. Genotyping-based genome-wide approaches

With the progress of genotyping technology, genome-wide association studies (GWAS) have evolved into efficient and effective tools for mapping genes underlying human phenotypes. This approach allows genotyping simultaneously thousands of SNPs across the whole genome. Although successful candidate gene studies have been performed in pharmacogenetics, they cannot identify genes outside our current knowledge. GWAS allow such novel discovery in an unbiased way (Motsinger-Reif et al., 2013).

Recent studies have demonstrated the utility of the GWAS approach for studying pharmacogenomic traits, including paclitaxel-induced neuropathy. The first CIPN GWAS included 855 subjects of European ancestry and the neuropathy was analyzed using both the cumulative dose analysis and the maximum peripheral neuropathy grade observed during treatment. They found a SNP in *FGD4* associated with the onset of sensory peripheral neuropathy in the discovery cohort (rs10771973; HR=1.57, 95%CI=1.30-1.91, $P=2.6 \times 10^{-6}$) and was replicated in additional 154 Europeans (HR=1.72, 95%CI=1.06-2.80, P=0.013) and 117 African Americans (HR=1.93, 95% CI=1.13-3.28, $P=6.7 \times 10^{-3}$). There was also evidence of association with the onset or severity of paclitaxel-induced sensory for *EPHA5* (rs7349683, HR=1.63, 95%CI=1.34-1.98, $P=9.6 \times 10^{-7}$) and *FZD3* genes ((rs7001034; OR=0.57, 95%CI=0.48-0.69, $P=3.1 \times 10^{-9}$). All these genes encode proteins that have been related with neuronal processes or diseases. FGD4 is involved in the regulation of actin cytoskeleton and cell shape, and mutations in the gen have been associated with Charcot-Marie-Tooth disease. EPHA5 is an ephrin receptor which plays crucial roles in several neuronal processes, and FZD3 has been associated with psychosis and congenital hydrocephalus (Baldwin et al., 2012).

In our group, a GWAS was performed with 144 white European patients treated with paclitaxel/ carboplatin and using the cumulative dose of paclitaxel up to the development of grade 2 sensory neuropathy.

We found a strong evidence of association for a gene encoding *EPHA4* locus (rs17348202, P=1.0x10^{-6}). Moreover, *EPHA6* and *EPHA5* were among the top hits (rs301927, P=3.4x10^{-5} and rs1159057, P=6.8x10^{-5}, respectively). A meta-analysis performed for *EPHA5*-rs7349683, the top marker for paclitaxel-induced neuropathy in Baldwin *et al* GWAS (in high linkage disequilibrium with rs1159057), was significant at genome wide level (HR=1.68, 95%CI=1.42-1.99, P=1.4x10^{-9}). This study provides independent confirmation of *EPHA5*-rs7349683 and identifies for the first time, in an unbiased manner, a marker of risk of paclitaxel-induced neuropathy with genome-wide significance. It also suggests that other *EPHA* genes may play an important role in the development of this toxicity (Leandro-García et al., 2013). Recently, Boora *et al* also were also able to replicate the association of *EPHA5* in a genotyping study including 119 paclitaxel-treated patients (OR=2.07, P=0.02) (Boora et al., 2016).

Another GWAS was conducted with 2,204 patients treated with paclitaxel with the aim of identifying SNPs associated with time to the first report of grade 2 to 4 neuropathy. Significant associations were found for *RWDD3* gene (rs2296308) and *TECTA* gene (rs1829), both associated with increased risk of neuropathy (HR=1.5; P=8.5 ×10^{-8} and HR=2.1; P=3.2×10^{-7}, respectively) (Schneider et al., 2011). However, another group failed to replicate these associations, and unexpectedly homozygous carriers of the variant allele in *TECTA* suggested protection rather than susceptibility to neuropathy (Bergmann et al., 2013). These contradictory results suggest important differences among the studies (e.g. different methodologies in the assessment of neuropathy or the use of paclitaxel alone versus paclitaxel plus carboplatin regimens).

6.2. Next Generation Sequencing

NGS has revolutionized the field of human genetics for both Mendelian disorders and complex traits (Ng et al., 2010, Panoutsopoulou et al., 2013). Nowadays, NGS is a feasible and cost-effective tool that allows the detection of both common variants and low-frequency/ rare variants associated with diseases (Cirulli and Goldstein, 2010, Shendure and Ji, 2008, Shendure, 2011)**.**

6.2.1. NGS–based candidate gene approaches

NGS can be used to perform candidate gene approaches by enrichment of a set of genes/ regions and exclusively performing sequencing in them. During the last part of the Thesis we adopted this technology to investigate candidate genes associated with paclitaxel-induced neuropathy. In addition to previously suggested candidates, we hypothesized that CMT genes may harbor non-pathogenic genetic variants that, while not being pathogenic, may predispose to acquired forms of polyneuropathies such as those caused by chemotherapeutic agents.

While performing this last project, Beutler *et al* published a study sequencing 49 CMT genes in 119 patients with different phenotype distribution (patients with high neuropathy vs. no/mild neuropathy). They identified an overrepresentation of rare non-synonymous coding variants in periaxin gene (*PRX*) in patients with high neuropathy but not in controls ($P=8x10^{-3}$), and showed that *ARHGEF10*, encoding rho guanine nucleotide exchange factor 10, was associated with paclitaxel-induced neuropathy ($P=5x10^{-4}$) due to the contribution of three common SNPs (i.e. rs9657362, rs2294039, and rs17683288). Of them, rs9657362 had the strongest effect (OR=4.8, $P=4x10^{-4}$). Interestingly, the majority of the rare variants identified in *PRX* were in exon 7, where the majority of known CMT mutations are found. In another study by the same group, they validate the association of *ARHGEF10* with paclitaxel-induced neuropathy (P=0.024). As in the original study, the strongest association was seen for rs9657362 (OR=3.56, P=0.018).

Thus, only one NGS study, focused exclusively on CMT, has been published. However, the most relevant genes identified so far in relation with paclitaxel-induced neuropathy remained unexplored with this technique.

6.2.2. NGS-based genome-wide approaches

NGS offers the possibility to study not only candidate genes but also to sequence the entire genome (whole genome sequencing, WGS) or all protein coding genes (whole exome sequencing, WES). However, previous to this Thesis no article had used WGS or WES in relation with drug-induced neuropathy.

6.3. Summary of genetic variants associated with CIPN

Several markers have been associated with paclitaxel-induced peripheral neuropathy; however, the evidences supporting these associations vary and there are also contradictory results. Possible explanations for the discrepancies among studies and the lack of replication for the majority of the neuropathy markers identified so far include: inadequate sample sizes, dissimilar scales and methods used to assess neuropathy, and non-homogenous treatment regimens across cohorts. Moreover, only common polymorphisms have been studied so far and the role of low-frequency variants in the susceptibility to suffer from neuropathy has not been explored, except for one study in which only CMT genes were sequenced (Beutler et al., 2014).

Currently, the most consistent associations with paclitaxel-induced neuropathy correspond to *CYP2C8*3* and *EPHA5*, based on evidences from several studies (Leskelä et al., 2011, Hertz et al., 2013, Baldwin et al., 2012, Leandro-García et al., 2013), (Hertz et al., 2014, Boora et al., 2016) (Figure 4).

Therefore, further investigation is needed to identify reliable markers contributing to paclitaxel-induced peripheral neuropathy. This would aid in the development of personalized paclitaxel treatments reducing severe cases of neuropathy.

In this Thesis we have identified and characterized genetic markers predictive of paclitaxel-induced peripheral neuropathy, using a variety of approaches: genotyping, whole exome sequencing and targeted next generation sequencing.

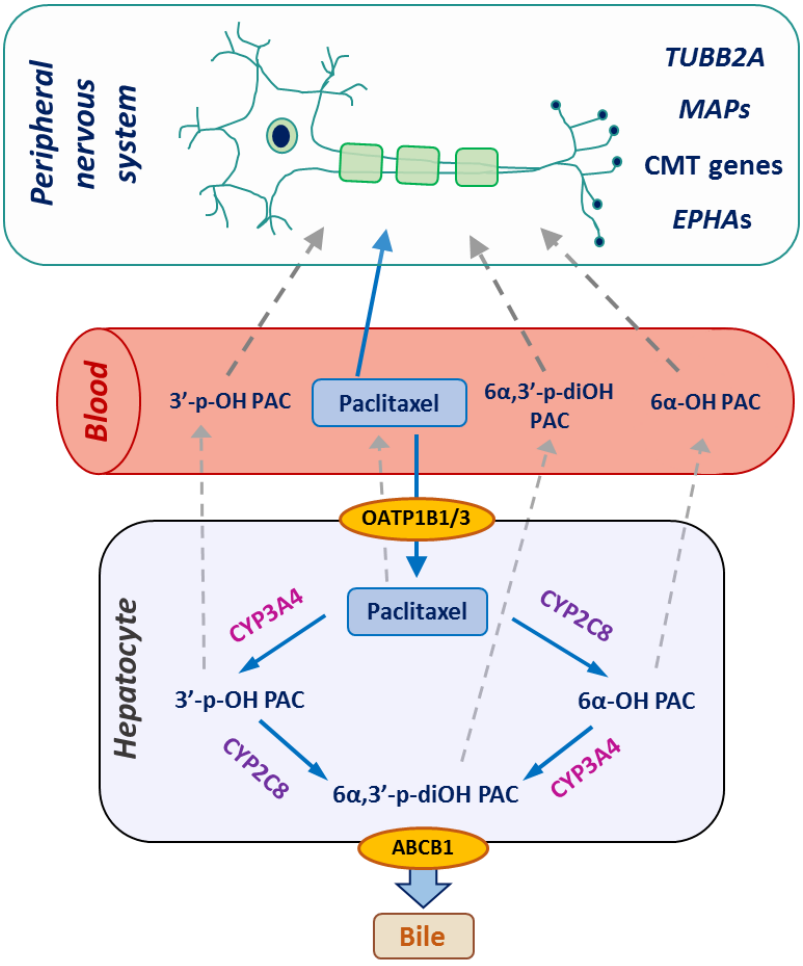

Figure 4. Paclitaxel pharmacokinetics and pharmacodynamics. 6α-OH PAC, 3'-p-OH PAC and 6α,3'-p-diOH PAC represent 6α-hydroxypaclitaxel, 3′-hydroxypaclitaxel and 6α-p-3'-dihydroxypaclitaxel paclitaxel metabolites.

OBJECTIVES

Although it has been proposed that there is a strong genetic component underlying the inter-individual variability in paclitaxel-induced neuropathy, currently, there are no markers used in the clinic that can identify, prior to the treatment, those patients with a high risk of this toxicity. Therefore, the main goal of this Thesis was to identify genetic markers predictive of peripheral neuropathy caused by paclitaxel, with the overall aim to provide with critical data that can help improve cancer therapy. The specific objectives of this Thesis are:

1) To explore the association of common single nucleotide polymorphisms, previously proposed by our group, as predictive markers of paclitaxel-induced neuropathy. Specifically, to study in depth *CYP2C8*3* variant and top signals identified in genome wide association studies (GWAS).

2) To identify novel low frequency genetic markers associated with increased susceptibility of paclitaxel-induced peripheral neuropathy. For this purpose we will apply whole exome sequencing and targeted next generation sequencing using highly informative selected series of patients. In addition, we will characterize the functional impact and world-wide distribution of the newly identified genetic variants.

ARTICLES

ARTICLE 1: Role of cytochrome P450 2C8*3 (*CYP2C8*3*) in paclitaxel metabolism and paclitaxel-induced neurotoxicity.

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Abstract:

Chemotherapy-induced peripheral neuropathy is an important clinical problem. Several anticancer drugs such as taxanes cause a severe and disabling neuropathy in a high proportion of patients. Therefore, the identification and validation of markers predictive of this neuropathy are an urgent need. The genetic background has been suggested to account for a high percentage of the inter-individual variability observed in neuropathy risk among paclitaxel-treated patients. Previous studies have identified polymorphisms in genes encoding paclitaxel metabolizing enzymes (*CYP3A4* and *CYP2C8*) as risk markers of the neuropathy.

*CYP2C8*3*, a frequent allele in the Caucasian population that encodes for a protein with two amino acid substitutions (p.R139K, p.K399R), has been suggested to be associated with paclitaxel neuropathy. However, there is contradictory data in the literature regarding the effect of *CYP2C8*3* on the metabolism of different substrates, *in vitro* and *in vivo*. We conducted a study in collaboration with Prof. Ingelman-Sundberg group at Karolinska Instituted to elucidate the implication of *CYP2C8*3* in paclitaxel metabolism by using a heterologous human cell expression system (HEK293 cells). To investigate the association of this variant with paclitaxel-induced neuropathy we used a large cohort of well-characterized cancer patients treated with this drug.

Concerning paclitaxel metabolism, CYP2C8.3 had a similar catalytic activity as CYP2C8 wild type when they were expressed in HEK293 cells for paclitaxel substrate.

Regarding *in vivo* data, we did not find a significant association between *CYP2C8*3* allele and maximum neuropathy grade in a cohort of 343 patients. However, a trend was observed when using the accumulated paclitaxel dose up to the development of grade 2 neuropathy, data available for 148 patients.

In conclusion, these results suggest that *CYP2C8*3* allele does not have a strong effect on paclitaxel metabolism *in vitro* neither on paclitaxel-induced neuropathy *in vivo*. Thus, *CYP2C8*3* genotyping is expected to have limited clinical utility to identify patients at risk of developing paclitaxel-induced neuropathy.

Personal contribution: I extracted DNA from the blood samples of the Spanish patients, performed *CYP2C8*3* genotyping and statistical analyses regarding *in vivo* data (logistic regression and Cox regression models). I contributed to the discussion of the results and helped drafting the paper.

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Pharmacogenomics

Role of cytochrome P450 *2C8*3* (*CYP2C8*3*) in paclitaxel metabolism and paclitaxel-induced neurotoxicity

Aim: The *CYP2C8*3* allele has been suggested as a risk factor for paclitaxel-induced neuropathy but the data hitherto published are conflicting. **Materials & methods:** In total 435 patients were investigated with respect to maximum neuropathy grade and accumulated paclitaxel dose. The enzymatic properties of CYP2C8.3 variant were analyzed using heterologous mammalian HEK293 cell expression system. **Results:** No significant association between *CYP2C8*3* allele and neuropathy was found, although a trend was observed. The paclitaxel and amodiaquine metabolism by CYP2C8.3 were found similar to CYP2C8.1, whereas CYP2C8.3 was more efficient in the metabolism of rosiglitazone. **Conclusion:** These results indicate a difference in substrate specificity between CYP2C8.1 and CYP2C8.3; however, the *CYP2C8*3* allele has no major impact on paclitaxel metabolism *in vitro* or of paclitaxel-induced neuropathy *in vivo*.

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Keywords: amodiaquine • breast cancer • CYP2C8 • neuropathy • ovarian cancer • paclitaxel • rosiglitazone

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Background

Paclitaxel is widely used alone or in combination with other cytotoxic drugs in breast, ovarian and lung cancers. Paclitaxel binds to β-tubulin and causes microtubule stabilization which impedes chromosome segregation, and results in cell apoptosis. Peripheral neuropathy and bone marrow suppression are the dose-limiting toxicities of paclitaxel. The severity of paclitaxel-induced peripheral neuropathy appears to be dependent on age, accompanying disorders, such as diabetes mellitus and alcoholism, but has also been suggested to be influenced by genetic factors, for example, by variability in genes encoding drug metabolizing enzymes [1–4]. Such polymorphisms are known to influence the pharmacokinetics and efficacy of many different drugs (cf. Sim and Ingelman-Sundberg, 2011 [5]).

Paclitaxel has been shown to be metabolized by two different cytochrome P450 enzymes, CYP2C8 and CYP3A4. CYP3A4 is a highly conserved gene, and there are few *CYP3A4* alleles, including *CYP3A4*22*, **20*, **25* and **27*, associated with an increased risk of developing paclitaxel-induced peripheral neuropathy [6,7]. *CYP2C8* polymorphisms include the *CYP2C8*3* allele with an allele frequency of 10–13% in the Caucasian populations, encoding a variant enzyme (CYP2C8.3) with two amino acid substitutions, Arg139Lys and Lys399Arg [8]. Green *et al.* and Bergmann *et al.* showed lower clearance of paclitaxel in patients being heterozygotes for *CYP2C8*3* (n = 23, n = 93, respectively) as compared with patients homozygous for *CYP2C8*1* [9,10] suggesting reduced metabolism of paclitaxel *in vivo* by CYP2C8.3. Recently, Leskelä *et al.* (2011) and Hertz *et al.* (2013, 2014) reported an association between *CYP2C8*3* and the accumulated dose of paclitaxel causing grade 2 neuropathy, with hazard ratios of 1.72 (p = 0.032; n = 118 patients), 1.93 (p = 0.006; n = 209) and 1.77 (p = 0.018; n = 412) [11–13], respectively. However, there are studies with

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opposite results showing lack of association between *CYP2C8*3* and taxane-induced neuropathy in large population studies [14–16].

From *in vitro* studies using other substrates, it has been suggested that CYP2C8.3 may cause reduced metabolism of the antimalarial drug amodiaquine [17], but increased metabolism of antidiabetic drug rosiglitazone [18]. However, Kaspera *et al.* showed that CYP2C8.3 metabolized these two substrates at higher rates as compared with CYP2C8.1 in an *Escherichia coli* system [19]. Due to such conflicting reports, the CYP2C8.3 enzymatic properties toward different substrates remain to be firmly established.

In order to shed more light on this controversial topic, we investigated the role of the *CYP2C8*3* allele in paclitaxel-induced neuropathy *in vivo* studying maximum paclitaxel-induced neuropathy grade and accumulated dose of paclitaxel until grade 2 neuropathy in patient cohorts of 343 and 148 patients, respectively. Furthermore, due to the previous inconsistent results on CYP2C8.3 activity mainly derived from nonmammalian systems, we have compared the enzymatic properties of CYP2C8.1 and CYP2C8.3 using a HEK293 cell expression system using paclitaxel, amodiaquine and rosiglitazone as substrates. The data indicate no major contribution of *CYP2C8*3* allele to both paclitaxel-induced neuropathy and CYP2C8.3 mediated paclitaxel metabolism *in vitro*. Similarly, CYP2C8.3 showed unaltered amodiaquine kinetics, but more efficient metabolism of rosiglitazone.

Materials & methods

Chemicals

Paclitaxel, rosiglitazone and NADPH were purchased from Sigma-Aldrich (MO, USA). Human CYP2C8+P450 reductase supersomes and 6α-hydroxypaclitaxel were acquired from BD Gentest (BD Bioscience, Franklin Lakes, NJ, USA). Anti-CYP2C8 purified MaxPab rabbit polyclonal (D01P) antibody was purchased from Abnova Taiwan Corporation (Taipei, Taiwan). A goat antirabbit IgG antibody conjugated with the horseradish peroxidase was purchased from Dako (Glostrup, Denmark) and the plasmid pcDNA™5/FRT vector was purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany).

Patients

Blood samples were collected from 304 breast (89%) and 39 ovarian (11%) cancer patients treated with paclitaxel in Spanish hospitals. Part of this series (236 patients) has already been communicated and the clinical characteristics have already been presented in [7]. Full characteristics for the 343 patients are presented in Supplementary Table 1 (see online at: www.futuremedicine.com/doi/suppl/10.2217/pgs.15.46). For each patient, the following information was collected: demographics, tumor characteristics, maximum neuropathy grade during paclitaxel treatment, cumulative dose of paclitaxel and paclitaxel dose reductions/suspensions and their causes. A neuropathy assessment was conducted in each patient to evaluate the maximum peripheral neuropathy grade during paclitaxel treatment as previously described [7]. In addition to maximum neuropathy grade, during paclitaxel treatment, accumulated paclitaxel dose up to grade 2 neuropathy was also available for 56 of the Spanish patients and also from 92 Danish patients previously published [10,20]. All cancer patients were at least 18 years of age, and the clinical information had been documented including histological cancer neoplasia, a life expectancy of greater than or equal to 12 weeks and Eastern Cooperative Oncology Group (ECOG) performance status smaller than or equal to two, adequate bone marrow, renal and hepatic function and no previous history of neuropathy, and usage of contraception. The recruitment of patients and collection of samples were approved by local internal ethical review committees (reference id: VF-20050083). All patients gave verbal and written informed consent to participate in the study.

DNA isolation & genotyping

FlexiGene DNA Kit (Qiagen, Germantown, MA, USA) was used to obtain genomic DNA from the blood samples of Spanish patients. Genotyping of *CYP2C8*3* allele (rs11572080, R139K) was performed on 15 ng of gDNA using the KASPar SNP Genotyping System (LGC Genomics, Teddington, Middelsex, UK). All assays included DNA samples with known genotypes and negative controls. The Sequence Detection System ABI PRISM® 7900HT (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used to determine fluorescence and for allele assignment.

The frequency of *CYP2C8*3* allele in the patients was similar to that previously reported in Caucasians [21] and the genotype distribution was in Hardy–Weinberg equilibrium.

Generation of CYP2C8 variants & overexpression of proteins in HEK293 cells

Human CYP2C8 cDNA was used to generate *CYP2C8*3* (416G>A and 1196A>G) sequence by using a QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene, CA, USA). The cDNAs were subcloned between the *Kpn*I and *ho*I restriction sites in the pCDNA5/FRT expression plasmid. Mutagen-

esis was confirmed by DNA sequencing. HEK293 cells were transiently transfected with these recombinant plasmids using Lipofectamine LTX and PLUS according to Invitrogen protocol in 150 mm culture dishes. HEK293 cells transfected with empty pcDNA5/FRT were used as a negative control.

Preparation of cell homogenates

Cell homogenates were prepared by resuspending pelleted cells in Tris-buffered saline (25 mM Tris base, 138 mM NaCl, 2.7 mM KCl, pH 7.4) that were then disrupted by 20 sonication 'bursts' each for 1 sec and centrifuged at 800 × *g* for 20 min at 4°C. The supernatant (S800 fraction) was used for western blot and enzyme kinetic assay. Total protein amount was determined by Bradford method [22].

Western blot

5–20 µg of protein from S800 fractions was separated by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Whatman® Optitran® reinforced nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) and probed with the anti-CYP2C8 purified MaxPab rabbit polyclonal antibody (Abnova, Taiwan) (1:200 dilution, incubated overnight at 4°C) or rabbit anti-ERp29 antibody (1:1000, incubated 1 h at room temperature). ERp29, an abundantly expressed endoplasmic reticulum escort chaperone was chosen as a loading control [23]. A goat antirabbit IgG antibody conjugated with the horseradish peroxidase was used as secondary antibody (1:5000, Dako Cytomation, Glostrup, Denmark) and incubated for 1 h at room temperature. The membranes were developed with SuperSignal West Pico Chemiluminescence kit (Pierce chemical, IL, US) and signals were visualized by the Fuji LAS-1000 gel documentation system (FujiFilm, Tokyo, Japan) . The relative expression levels of the CYP2C8 variant apoproteins were determined by densitometric analysis of protein bands using Image Gauge V4.0 software (FujiFilm, Tokyo, Japan). Different amounts (0.025–0.08 pmol) of CYP2C8 recombinant protein (BD Supersomes) were used for plotting the calibration curve. Experiments were repeated for 3-times.

HPLC analysis

Paclitaxel

S800 fractions, corresponding to 300 µg protein, from the transfected HEK 293 cells were incubated in a final volume of 0.1 ml of 50 mM potassium phosphate buffer, pH 7.4 at 37°C using 0-40 µM paclitaxel. The reaction was initiated by adding 1 mM NADPH and it was linear for 90 min. The reaction was stopped by adding 200 µl of ice-cold 100% methanol and samples were stored at -80°C until further analysis. After centrifugation at 16,000 × *g* for 10 min in cold room, the supernatant was injected into the C18 Spherisorb ODS-2; 5µm, 150 × 4.6 mm column (Waters, Milford, MA, USA). Metabolite and parent compound were separated by reverse phase HPLC with 65% methanol in isocratic mode with flow of 1 ml/min in the Waters 2690/996 and the signal was detected at UV 229 nm. Docetaxel was spiked in each sample (retention times of 6OH paclitaxel, paclitaxel and docetaxel = 13.9, 17.8 and 20.5 min).

Amodiaquine

The enzyme assay was performed similar to that of paclitaxel. The hydroxylation reaction was linear for 90 min. The catalytic reaction was stopped by adding 100 µl of ice-cold 100% acetonitrile containing 10 µM chloroquine. After centrifugation at 16,000 × *g* for 10 min at 4°C, supernatant was injected into the Zorbax SB C18 125 × 4.6 mm, 3.5 µm column (Agilent, Santa Clara, CA, USA). Metabolite and parent compound were separated by Varian ProStar 310 HPLC system using 16% methanol in 50 mM phosphate buffer, pH 2.1 as mobile phase with flow of 1 ml/min. The compounds were detected using UV 340 nm and the retention times of chloroquine, N-bis-desethyl-amodiaquine and amodiaquine were 7.6, 9.9 and 12.2 min, respectively.

Rosiglitazone

N-demethylation assay was performed in a final volume of 0.1 ml of 50 mM potassium phosphate at 37°C. The reaction was initiated by adding 1mM NADPH. N-demethylation was linear for 90 min. The reaction was stopped by adding 300 µl of ice-cold 100% methanol and all the samples were stored in freezer (-20°C) until further analysis. After addition of 10 µl of internal standard (1.1 µg/ml venlafaxine), the sample was centrifuged (2800 × *g*, 2°C, 5 min). 200 µl of the supernatant was diluted with 100 µl 0.1% formic acid and 20 µl of the solution analyzed on a Synergi 4µ MAX-RP 80A-column 100 × 3 mm, 4 µm with a precolumn Gemini C18 4 × 2.0 mm (both from Phenomenex, Torrance, CA, USA) with a flow rate of 0.9 ml/min at 9°C. The mobile phase consisted of acetonitrile : methanol : sodium acetate 0.01 M, pH 6 = 30:8:62 (V/V/V). Both N-demethyl-rosiglitazone (NDES) and rosiglitazone were detected using fluorescent detector with an excitation wavelength of 250 nm and monitoring the emission wavelength at 390 nm. Venlafaxine was detected using 230 and 310 nm for excitation and emission.

Statistical analysis

The kinetic constants, K_m and V_{max} were estimated using nonlinear regression analysis of Michaelis–Men-

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ten model by GraphPad Prism version 5.0 software (GraphPad Software, Inc., CA, USA). Amodiaquine and paclitaxel concentration-velocity curves were fitted by using nonlinear regression analysis of hill slope model by GraphPad Prism. Intrinsic clearance (CL_{int}) values were determined as the ratio of V_{max}/K_m. All values are expressed as the mean ± standard deviation of triplicates. Student *t* test was used to evaluate the difference between two variants' activities (GraphPad) indicating significance with p value ($p < 0.05$).

The association between maximum paclitaxel-induced neuropathy grade (ranked 0 to 3) and *CYP2C8*3* genotype was assessed using an ordinal logistic regression. Binary logistic regression was applied to evaluate the association between paclitaxel treatment modifications (binary variable) and *CYP2C8*3* genotype. Tumor type, total number of chemotherapy cycles and potential confounders (diabetes mellitus and alcoholism) were included as covariates in the multivariable analysis. Association between *CYP2C8*3* and neuropathy was also evaluated, analyzing the accumulated dose of paclitaxel up to the development of grade 2 neuropathy (cumulative dose analysis) using Cox regression analysis. Multivariable analysis included country of origin (in this analysis patients from both Spain and Denmark were included), tumor type and total number of chemotherapy cycles as covariates. SPSS software package v.19 (IBM Corp., Armonk, NY, USA) was used for these statistical analyses. p-values less than 0.05 were considered statistically significant.

Results

Influence of *CYP2C8*3* on the paclitaxel-induced neuropathy

A total of 343 paclitaxel-treated Spanish patients were recruited and genotyped for *CYP2C8*3*. Demographic and clinical data of these patients are presented in Supplementary Table 1. We examined the distribution of *CYP2C8*3* in the patients but did not find an association between the *CYP2C8*3* allele and maximum paclitaxel-induced neuropathy grade (Figure 1A). By analyzing paclitaxel treatment modifications due to neuropathy, again we did not observe an association with *CYP2C8*3* allele (Figure 1B). Multivariate analysis including relevant covariates did not change these results.

Cumulative dose analysis is an alternative method to test for neuropathy risk factors. Neuropathy symptoms occur after multiple chemotherapy cycles and therefore the cumulative dose of paclitaxel is a significant risk factor in chemotherapy-induced neuropathy. The accumulated paclitaxel dose causing grade 2 neuropathy was available from 56 of the 343 Spanish patients and similar data were also available from a previously published Danish study (n = 92) [10,20], resulting in a total of 148 patients that could be used for cumulative dose analysis (Figure 2). Cox regression analysis showed no statistically significant association between neuropathy and *CYP2C8*3* genotype, but a multivariate analysis including country of origin, tumor type and number of chemotherapy cycles as covariates, gave a trend toward higher neuropathy risk for carriers of the *CYP2C8*3* allele (HR: 1.70; 95% CI: 0.94–3.05; p = 0.078; Figure 2).

Enzymatic activity of CYP2C8.1 & CYP2C8.3 expressed in HEK293 cells

The capacity of the CYP2C8.1 and CYP2C8.3 enzyme variants to metabolize paclitaxel, amodiaquine and rosiglitazone was determined in a mammalian expression system using HEK293 cells. The apoprotein levels of recombinant CYP2C8 enzymes were determined using the densitometric quantification of immunoblots. As shown in Figure 3, the apoprotein levels of the two CYP2C8 variants were similar in the expression system used. The S800 fraction isolated from the transfected cells was incubated with paclitaxel, amodiaquine and rosiglitazone and the corresponding metabolites, 6 OH-paclitaxel, N-desethyl-amodiaquine and NDES were identified using substrate-specific HPLC assays.

As shown in Figure 4, the enzyme kinetic parameters for CYP2C8.1 and CYP2C8.3 dependent metabolism of paclitaxel and amodiaquine in this expression system were similar (no significant differences in K_m or CL_{int} for CYP2C8.1 and CYP2C8.3 for either paclitaxel or amodiaquine; $p > 0.05$). However, rosiglitazone was metabolized to NDES more extensively by CYP2C8.3 than by CYP2C8.1 (Figure 4 & Table 1). For rosiglitazone, the intrinsic clearance and the K_m by CYP2C8.3 were significantly different from that of CYP2C8.1 (1.8-fold higher and1.7-fold lower than CYP2C8.1, respectively), whereas both enzyme variants exhibited similar V_{max} values.

Discussion

Genetic variations in paclitaxel metabolizing enzymes have been considered as risk factors for paclitaxel-induced peripheral neuropathy. In particular, *CYP2C8*3* has been suggested to be associated with paclitaxel-induced adverse effects. However, in the current study encompassing a cohort of 343 patients, we did not detect an association between the *CYP2C8*3* allele and paclitaxel-induced neuropathy. In addition, the catalytic efficacy of CYP2C8.3 expressed in HEK293 cells did not differ from the one of CYP2C8.1 with respect to paclitaxel metabolism.

As mentioned, the association of the *CYP2C8*3* allele with higher risk for neuropathy is debated and

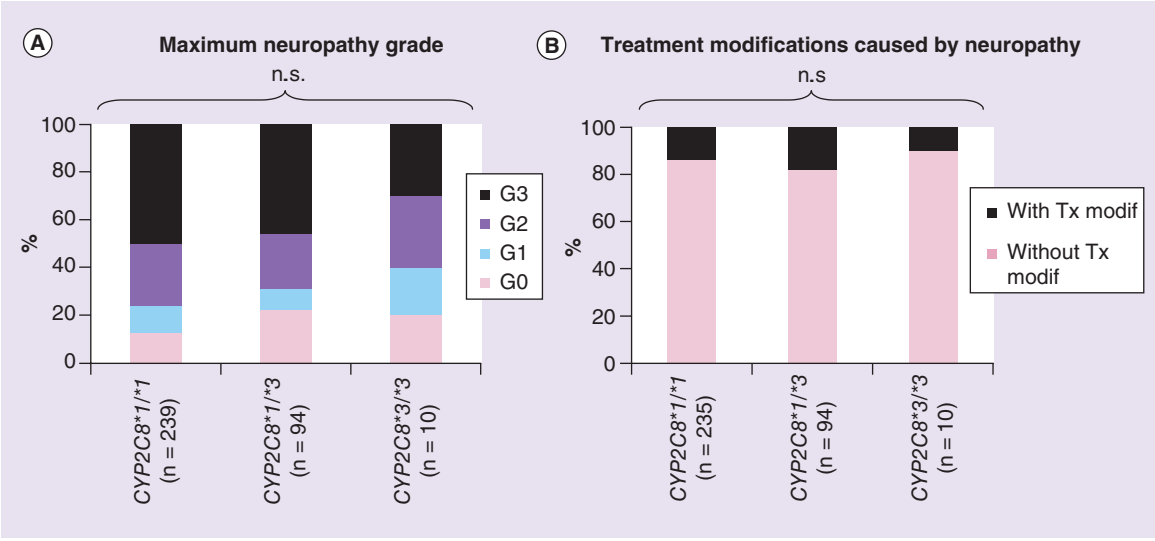

Figure 1. Paclitaxel-induced neuropathy grade and treatment modifications grouped by *CYP2C8*3* genotype. **(A)** Maximum neuropathy grade and **(B)** treatment modifications due to neuropathy were compared among patients with different *CYP2C8*3* genotypes. As described in the 'Materials & methods' section, ordinal logistic regression or binary logistic regression, respectively, were performed. 'Tx modif' refers to treatment modifications due to peripheral neuropathy.

constitutes a controversial issue. Some clinical investigations report such association [9,11–13,24] in line with other studies indicating lower paclitaxel clearance [9–10,25]. However, here we did not observe higher neuropathy grades in *CYP2C8*3* carriers (Figure 1) in agreement with other studies [6,14,26–30]. In the cumulative dose analysis, we found a trend toward higher paclitaxel-induced neuropathy risk in *CYP2C8*3* carriers (HR: 1.70, p = 0.078; Figure 2). In line with this result, recent large studies by Abraham *et al.* [15] and Baldwin *et al.* [16] (>800 patients), found no statistically significant association of *CYP2C8*3* variant allele with taxanes related sensory neuropathy using cumulative dose analysis (HR: 1.22; p = 0.14 and HR: 1.21; p = 0.23, respectively).

Although CYP2C8 constitutes a major metabolic pathway of paclitaxel, CYP3A4 also contributes to its metabolism. In fact, in a recent study, defective *CYP3A4* variants, including *CYP3A4*20*, *CYP3A4*25* and *CYP3A4*27* were found to be associated with a higher risk of paclitaxel-induced neuropathy [7]. Thus, carriers of these *CYP3A4* variants had more severe neuropathy and higher probability of neuropathy-induced paclitaxel treatment modifications (2- to 7-fold higher risk) as compared with patients with wild-type CYP3A4 [7]. Increased paclitaxel neuropathy risk has also been reported for *CYP3A4*22* allele, which causes decreased expression of the CYP3A4 enzyme [6,31]. The neuropathy risk (HR: 22.1, p < 0.001) [6] shown in this study for carriers of *CYP3A4*22* allele is substantially higher than the HR value for *CYP2C8*3* in the present study (HR: 1.70, p = 0.078) and also when compared with the risk for *CYP2C8*3* reported in previous reports

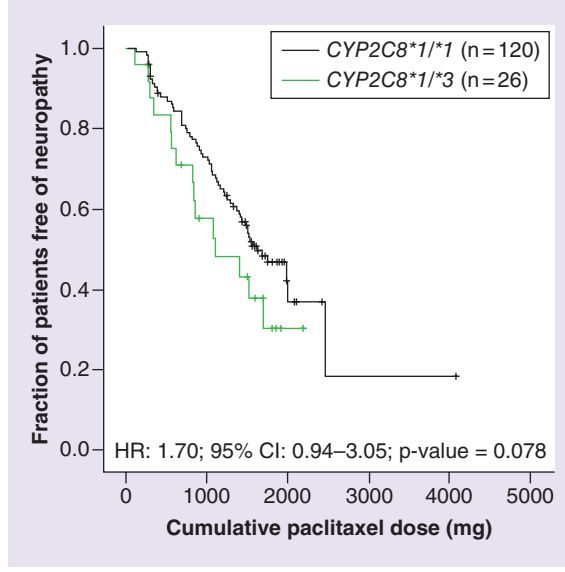

Figure 2. Kaplan–Meier curve comparing cumulative paclitaxel dose up to grade 2 neuropathy by *CYP2C8*3* genotype. A total of 148 paclitaxel-treated patients were grouped according to *CYP2C8*3* genotype and compared with paclitaxel cumulative dose up to the development of grade 2 peripheral neuropathy. p-values correspond to multivariate Cox regression analysis, including country of origin, tumor type and number of chemotherapy cycles as covariates. The curves, HR, 95% CI and p-values presented correspond to the comparison between *CYP2C8*1/*3* and *CYP2C8*1/*1* patients. Only two patients were *CYP2C8*3/*3* and are not included in the graph. HR: Hazard ratio.

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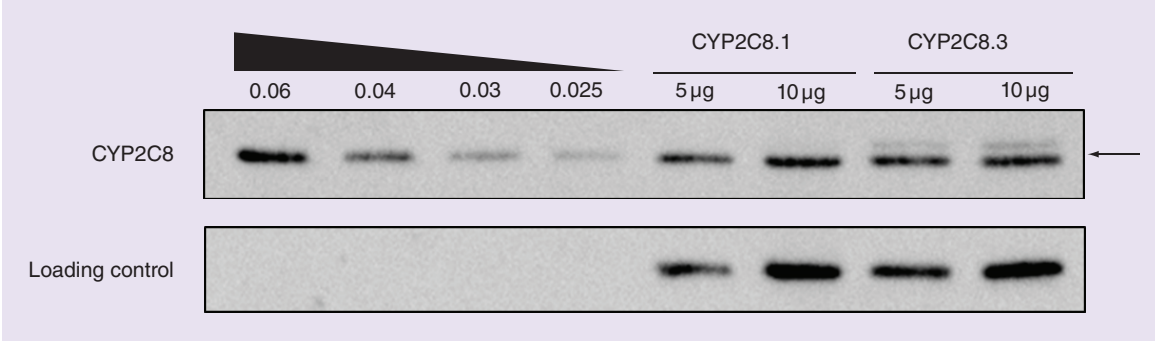

Figure 3. Protein expression of CYP2C8.1 and CYP2C8.3. S800 fractions prepared from transiently transfected HEK293 cells were immunoblotted with antibodies for CYP2C8 and loading control, ERp29 [23]. A representative image is shown for CYP2C8 variants with samples loaded in two concentrations. 0.025–0.08 pmol of CYP2C8 recombinant protein (BD Supersomes) was used for plotting the calibration curve. Densitometric analysis estimated 7.5 ± 0.5 pmol/mg protein for CYP2C8.1 and 7.8 ± 0.7 pmol/mg protein for CYP2C8.3.

(HR between 1.72 and 1.93) [11–13]. These data may indicate a stronger effect of the identified *CYP3A4* defective variants than of *CYP2C8*3* polymorphism in paclitaxel-induced neuropathy.

The reason for the conflicting data regarding catalytic activity of CYP2C8.3 on the metabolism of paclitaxel might be associated with the type of expression system used. Heterologously expressed enzymes fold differently in different types of cells. Thus for example, CYP2D6.17 identified in a patient possessing defective drug metabolism showed identical rate of metabolism as the CYP2D6.1 enzyme using bufuralol as a substrate when the enzyme was expressed in yeast cells but showed much reduced hydroxylation rate compared with CYP2D6.1 when expressed in COS-1 cells in accordance with the *in vivo* data [32]. Similarly lower intrinsic paclitaxel clearance as compared with CYP2C8.1 has been observed when CYP2C8.3 was expressed in heterologous systems based on *E. coli*, yeast or insect cells [8,19,33,34], whereas when paclitaxel hydroxylation was evaluated in mammalian systems using transfected human hepatoma cells HepG2 [35] or in HEK293 cells, as reported here, the kinetics of paclitaxel metabolism by CYP2C8.3 and CYP2C8.1 were similar. The differences above might thus be inherited in aberrant folding or interaction with redox partners in the heterologous expression systems used.

The enzymatic activity of CYP2C8.1 and CYP2C8.3 proteins was also tested using amodiaquine and rosiglitazone as substrates. For amodiaquine, we did not observe differences in kinetic parameters between the enzyme variants, although there are previous conflicting results for CYP2C8.3 in *E. coli* [17,19]. Concerning rosiglitazone, we found a higher clearance for CYP2C8.3, which is in accordance with other *in vitro* [19] and *in vivo* studies [18,36].

The CYP2C8.3 variant enzyme contains two conserved amino acid changes, R139K and K399R. Therefore, it could *a priori* be suggested that the physicochemical properties of CYP2C8.3 should not differ much from CYP2C8.1, and *in silico* analysis using two online tools PolyPhen2 [37] and SIFT [38] support this

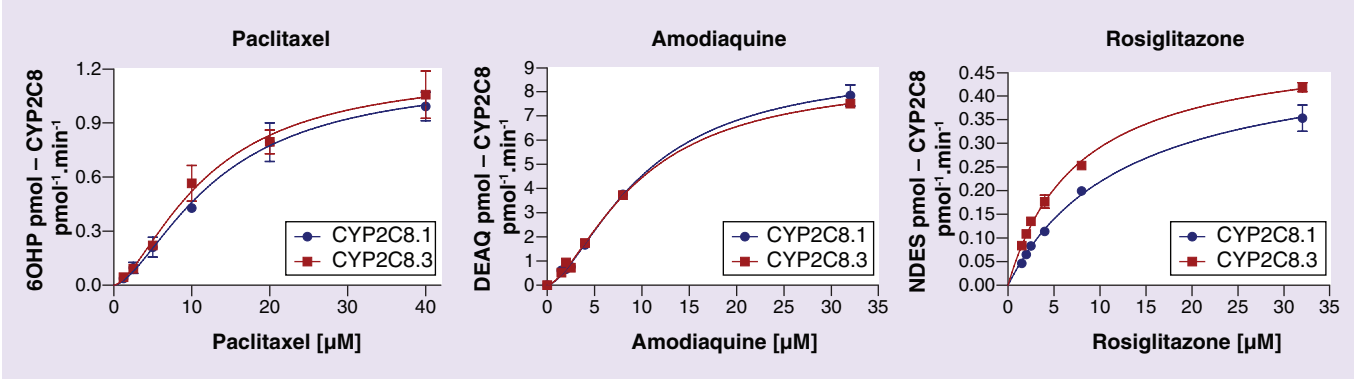

Figure 4. Kinetic evaluation of CYP2C8.3 on paclitaxel 6α-hydroxylation, amodiaquine N-de-ethylation and rosiglitazone ρ-hydroxylation. The activity values were normalized by the corresponding amounts of CYP2C8 variant proteins as estimated by densitometry of western blot bands (Figure 1). Curve fitting was accomplished by GraphPad v. 5.0 using H-loop (paclitaxel and amodiaquine) and Michaelis–Menten plot (rosiglitazone).

Table 1. Kinetic constants of reconstituted CYP2C8.1 and CYP2C8.3 with different substrates.

		K_m (μM)	V_{max} (pmol CYP2C8 $pmol^{-1}$ min^{-1})	CL_{int} (V_{max}/K_m)
Paclitaxel	CYP2C8.1	13.11 ± 2.01	1.17 ± 0.10	0.089 ± 0.005
	CYP2C8.3	11.87 ± 2.11	1.20 ± 0.12	0.101 ± 0.007
Amodiaquine	CYP2C8.1	10.04 ± 1.11	9.08 ± 0.49	0.905 ± 0.046
	CYP2C8.3	9.67 ± 0.77	8.66 ± 0.32	0.896 ± 0.036
Rosiglitazone	CYP2C8.1	12.75 ± 1.10	0.49 ± 0.02	0.039 ± 0.002
	CYP2C8.3	7.67 ± 0.47*	0.52 ± 0.01	0.067 ± 0.002*

All experiments were carried out in triplicates and the data are presented as mean ± standard error of the mean.
*$p < 0.05$ as compared with CYP2C8.1.

notion (results not shown). Such predictions are in good agreement with the experimental data showing no difference in catalytic activities of these two variant proteins toward paclitaxel and amodiaquine. Interestingly, an *in silico* study based on the crystal structure of CYP2C8 complexed with troglitazone, a drug similar to rosiglitazone, predicts a missing salt bridge in R139K mutant whereas the salt bridge in K399R should remain intact. The authors conclude that this may affect the substrate specificity of the R139K protein [19], and this can be suggested as a possible explanation for the different rosiglitazone conversion rates found in our study. Different enzyme kinetics for two different substrates has previously been shown for other P450s, for example for CYP2D6.17 variant for codeine and bufuralol [32].

Paclitaxel-induced neurotoxicity remains a major detrimental factor in ovarian/breast cancer chemotherapy and the mechanisms of such phenomenon are still unclear. Our study indicates that variations in the *CYP2C8* gene do not affect the metabolism of paclitaxel. The impact of *CYP2C8*3* allele seems to be moderate in neurotoxicity given that cumulative dose analysis only showed a weak nonsignificant trend.

Conclusion & future perspective

In conclusion, using two patient cohorts with a total of 435 cases, we found no major impact of the *CYP2C8*3* allele on the risk of paclitaxel-induced neuropathy (HR: 1.70, p = 0.078). This is supported by our *in vitro* findings. Future work should be directed at revealing additional genetic factors associated with paclitaxel-induced neuropathy.

Financial & competing interests disclosure

This study was supported by grants from The Swedish Research Council, The Swedish Cancer Society, the County Council in Östergötland, the Spanish Ministry of Economy and Competiveness (SAF2012-35779) and the Danish Ministry of Interior Affairs and Health (2001-2007) (J. nr 2006-12103-276) and the Danish Research Agency (J. nr 271-05-0266). M Apellániz-Ruiz is a predoctoral fellow of 'la Caixa'/CNIO In-

Executive summary

Background

- Paclitaxel is a widely used anticancer drug that frequently causes peripheral neuropathy.
- CYP2C8 is the main paclitaxel metabolizing enzyme and *CYP2C8*3* allele has been suggested as a risk factor for paclitaxel-induced neuropathy.
- However, the hitherto published *in vivo* and *in vitro* data are highly controversial with respect to the influence of *CYP2C8*3*.

Materials & methods

- Association between *CYP2C8*3* genotype and paclitaxel-induced neuropathy was investigated in 435 cancer patients.
- The impact of CYP2C8.3 on paclitaxel metabolism was analyzed using a HEK293 cell expression system. Also amodiaquine and rosiglitazone were analyzed in the same manner.

Results

- No statistically significant association between *CYP2C8*3* and paclitaxel-induced neuropathy was found.
- The CYP2C8.3 enzyme kinetics of paclitaxel and amodiaquine were similar to CYP2C8.1, whereas CYP2C8.3 was more efficient in the conversion of rosiglitazone.

Conclusion

- No major impact of *CYP2C8*3* allele on paclitaxel-induced neuropathy was found, consistently with no changes in *in vitro* activity of CYP2C8.3 toward paclitaxel as compared with CYP2C8.1.

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ternational PhD programme. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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SUPPLEMENTARY MATERIAL:

Supplementary Table1. Characteristics of the 343 paclitaxel-treated patients

Characteristics	N (%)*
Age (years)	
Median (min-max)	52 (30-82)
Tumor type	
Breast	304 (89%)
Ovary	39 (11%)
Type of paclitaxel treatment	
First line	336 (98%)
Second line[a]	7 (2%)
Paclitaxel chemotherapy treatment[b]	
FEC+T[c]	146 (43%)
AC+T[d]	70 (20%)
T+FEC[e]	51 (15%)
C+T[f]	40 (12%)
T+AC[g]	17 (5%)
Others	19 (5%)
Number of paclitaxel cycles	
(min-max)	8 (3-44)
Maximum sensory neuropathy grade[h]	
G0	53 (16%)
G1	38 (11%)
G2	86 (25%)
G3	166 (48%)
Treatment modifications[i]	
Due to neuropathy	50 (15%)
Due to all causes	77 (22%)

* Unless otherwise stated

[a] Patients with second line paclitaxel treatment and no previous cytotoxic drugs in first line therapy.

[b] Some patients receiving chemotherapeutic drugs in combination with targeted therapy (bevacizumab, trastuzumab, denosumab or pertuzumab) are included in the table according to the chemotherapy agents received.

[c] FEC+T: 5-fluorouracil 600 mg/m2, epirubicin 90 mg/m2 and cyclophosphamide 600 mg/m2, every 21 days, followed by paclitaxel 80 or 100 mg/m2, every 7 days.

[d] AC+T: doxorubicin 60mg/m2 and cyclophosphamide 600 mg/m2, every 21 days, followed by paclitaxel 80mg/m2, every 7 days.

[e] T+FEC: paclitaxel 80 mg/m2, every 7 days, followed by 5-fluorouracil 600 mg/m2, epirubicin 90 mg/m2 and cyclophosphamide 600 mg/m2, every 21 days.

[f] C+T: carboplatin AUC5-6 and paclitaxel 175mg/m2, every 21 days.

[g] T+AC: paclitaxel 80mg/m2, every 7 days, followed by doxorubicin 60mg/m2 and cyclophosphamide 600 mg/m2, every 21 days.

[h] NCI-CTC v4.

[i] Considering as treatment modifications paclitaxel dose reductions or treatment suspensions.

ARTICLE 2: Replication of Genetic Polymorphisms Reported to Be Associated with Taxane-Related Sensory Neuropathy in Patients with Early Breast Cancer Treated with Paclitaxel—letter.

Authors: **Apellániz-Ruiz M**, Sánchez-Barroso L, Gutiérrez-Gutiérrez G, Sereno M, García-Donás J, Åvall-Lundqvist E, Gréen H, Brøsen K, Bergmann TK, Rodríguez-Antona C.

Published in Clin Cancer Res. 2015 Jul 1;21(13):3092-3

Abstract:

Candidate gene approaches have allowed to identify common polymorphisms in genes involved in paclitaxel pharmacokinetics and pharmacodynamics; however, with the development of genome-wide association studies (GWAS) millions of SNPs across the whole genome can be genotyped simultaneously. GWAS allow to identify new genes explaining the basis of human traits such as drug-induced toxicity. Two previous GWAS carried out by us and by another group, suggested polymorphisms in ephrin type-A receptors (encoded by *EPHA* genes) as predictors of paclitaxel-induced neuropathy.

In this study we evaluated the association of 4 common SNPs in *EPHA4*, *EPHA5*, *EPHA6* and *EPHA8*, with paclitaxel-induced peripheral neuropathy. To accomplish this goal, we collected a series of 146 patients treated with paclitaxel and with neuropathy data recorded cycle by cycle. We found that the SNPs in *EPHA5*, *EPHA6* and *EPHA8* genes were associated with a higher risk to suffer peripheral neuropathy. Regarding the SNP in *EPHA4*, we had low statistical power to detect an association due to the low frequency of the variant allele.

Moreover, as EphA receptor tyrosine kinases are involved in a variety of neuronal-related functions, including neural repair after injury, the findings herein described not only validate the involvement of EphA receptors in paclitaxel-induced neuropathy but they also suggest their potential role as neuropathy risk markers for other neurotoxic drugs.

Personal contribution: I participated in the design of the study. I performed the genotyping of all SNPs in the samples and carried out the subsequent statistical analyses using Kaplan–Meier and Cox regression models. Finally, I contributed to the discussion of the results and I was the principal author drafting the paper.

Clinical Cancer Research

Replication of Genetic Polymorphisms Reported to Be Associated with Taxane-Related Sensory Neuropathy in Patients with Early Breast Cancer Treated with Paclitaxel—Letter

María Apellániz-Ruiz[1], Lara Sánchez-Barroso[1], Gerardo Gutiérrez-Gutiérrez[2], María Sereno[3], Jesús García-Donás[4], Elisabeth Åvall-Lundqvist[5], Henrik Gréen[6,7,8], Kim Brøsen[9], Troels K. Bergmann[9], and Cristina Rodríguez-Antona[1,10]

We have with great interest read the pharmacogenetic study by Abraham and colleagues (1) reporting SNPs associated with paclitaxel-induced neuropathy. The identification of markers predictive of sensory neuropathy is an important clinical problem for taxanes, vinca-alkaloids, platinum compounds, bortezomib, and thalidomide, among other anticancer drugs. In this respect, the study by Abraham and colleagues is a remarkably large study investigating 73 SNPs previously associated with taxane-related sensory neuropathy (TRSN) in 1,303 European individuals treated with paclitaxel (1). The authors found significant results for nine SNPs, including *EPHA6*-rs301927. Two genome-wide association studies (GWAS; refs. 2, 3) suggest *EPHA5*-rs7349683 as a neuropathy marker (meta-analysis *P* value of 1.4×10^{-9}), and in our study, other members of the Eph receptor family members were also associated with paclitaxel-induced neuropathy (3).

To follow up our initial results suggesting that ephrin type A receptors are important factors influencing TRSN, we analyzed detailed neuropathy data, recorded cycle by cycle using the NCI-CTCAE, from 146 patients treated with first-line paclitaxel. Patients had either ovarian (72%) or breast cancer; 57 (39%) were prospectively recruited in Spain and 89 patients were from a previously described Danish cohort (4). The study was approved by the corresponding ethical review committees and was carried out in accordance with the Helsinki declaration. We genotyped 4 SNPs in *EPHA4*, *EPHA5*, *EPHA6*, and *EPHA8* genes (rs17348202, rs7349683, rs301927, and rs209709, respectively) and 3 SNPs in *XKR4*, *PIK3IP1*, and *SGCG* genes (rs4737264, rs5749248, and rs1753097, respectively), all top signals in our GWAS (3). When tested against TRSN using a cumulative dose analysis, all SNPs in *EPHA* genes, except for *EPHA4*-rs17348202 (minor allele frequency = 0.05, indicating low statistical power), were associated with an increased neuropathy risk (Fig. 1). When analyzing the SNPs using maximum neuropathy grade, only *EPHA6*-rs301927 showed a trend toward increased toxicity ($P = 0.069$), suggesting

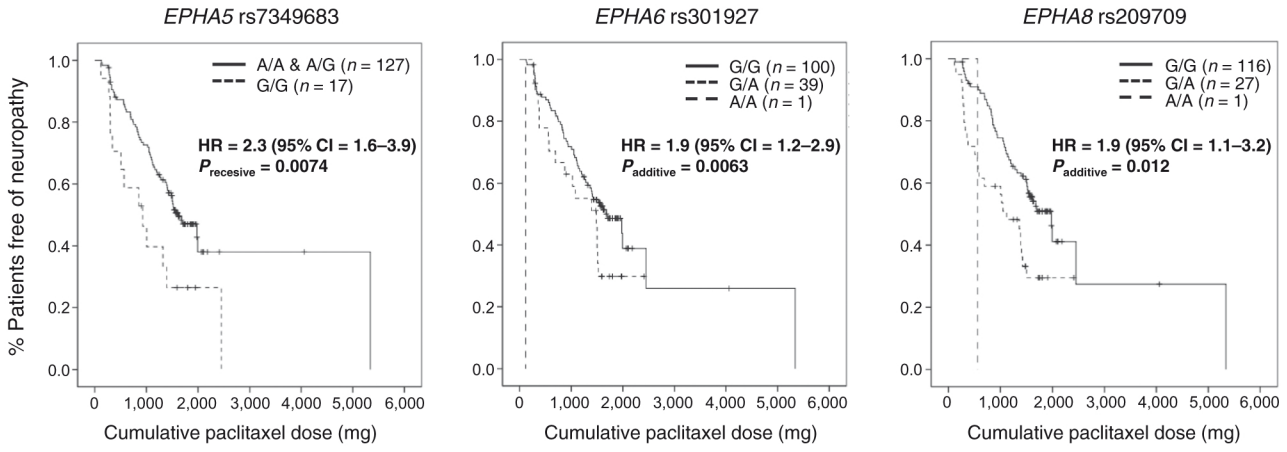

Figure 1.
Kaplan–Meier comparisons by *EPHA* SNPs. Paclitaxel-treated patients grouped according to *EPHA5*-rs7349683, *EPHA6*-rs301927, and *EPHA8*-rs209709 and compared with the cumulative dose of paclitaxel up to the development of grade 2 peripheral sensory neuropathy. *P* values correspond to Cox regression analysis including country as covariate; results from rs7349683 correspond to recessive genetic model.

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that cumulative dose analysis is more sensitive to detect differences in neuropathy. No evidence of association was found for SNPs in other genes.

From a biologic perspective, Eph receptors represent a family of receptor kinases, involved in axon guidance and other neural-related functions, such as neuronal regeneration following nerve injury (5). Thus, this prospective study, together with that from Abraham and colleagues and previous reports, supports an increased TRSN risk for *EPHA5*-rs7349683 (2, 3), *EPHA6*-rs301927 (1, 3), and *EPHA8*-rs209709 (3). Furthermore, because EPHA proteins mediate neural injury repair, these SNPs could act as broad-spectrum neuropathy risk markers relevant for many neurotoxic drugs. Abraham and colleagues performed an exhaustive study of SNPs previously associated with TRSN; however, in view of these results, it would be interesting if the authors could further investigate these potentially clinically relevant markers (e.g., *EPHA8*-rs209709 and *EPHA5*-rs7349683 under different genetic models).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by projects from the Spanish Ministry of Economy and Competiveness (grant number SAF2012–35779), the Danish Ministry of Interior Affairs and Health (2001-2007; J.nr 2006-12103-276), the Danish Research Agency (J.nr 271-05-0266), and the Swedish Research Council and the Swedish Cancer Society. María Apellániz-Ruiz is a predoctoral fellow of "la Caixa"/CNIO international PhD programme.

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Clinical Cancer Research

Correction: Replication of Genetic Polymorphisms Reported to Be Associated with Taxane-Related Sensory Neuropathy in Patients with Early Breast Cancer Treated with Paclitaxel—Letter

In this letter (Clin Cancer Res 2015;21:3092–3), which was published in the July 1, 2015, issue of *Clinical Cancer Research* (1), the A/A and G/G labeling in each panel of Fig. 1 is incorrect—the labels should be reversed. A corrected version of the figure is shown below. The figure legend and main text remain unchanged. The error does not affect the conclusions set forth in the letter. The authors regret this error.

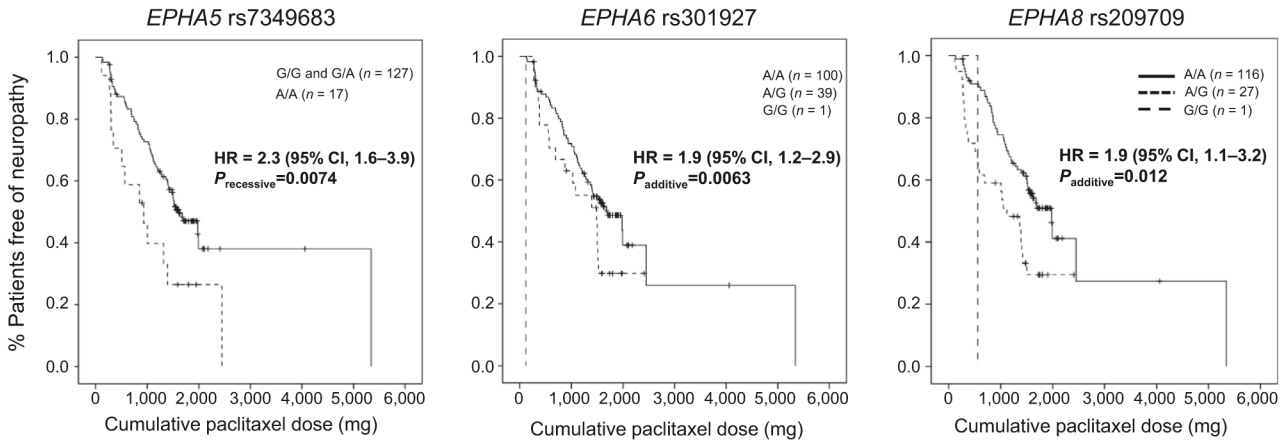

Figure 1.

Reference

1. Apellániz-Ruiz M, Sánchez-Barroso L, Gutiérrez-Gutiérrez G, Sereno M, García-Donás J, Åvall-Lundqvist E, et al. Replication of genetic polymorphisms reported to be associated with taxane-related sensory neuropathy in patients with early breast cancer treated with paclitaxel—letter. Clin Cancer Res 2015;21:3092–3.

Published online September 15, 2015.
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ARTICLE 3: Whole-exome sequencing reveals defective *CYP3A4* variants predictive of paclitaxel dose-limiting neuropathy.

Authors: **Apellániz-Ruiz M**, Lee MY, Sánchez-Barroso L, Gutiérrez-Gutiérrez G, Calvo I, García-Estévez L, Sereno M, García-Donás J, Castelo B, Guerra E, Leandro-García LJ, Cascón A, Johansson I, Robledo M, Ingelman-Sundberg M, Rodríguez-Antona C.

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Abstract:

The neuropathies triggered by the anticancer drug paclitaxel can lead to dose reductions or even treatment suspension and, in severe cases, it can cause irreversible nerve damage. In this regard, the selection of phenotypic outliers has been shown to be a powerful strategy to identify genetic variants associated with drug outcomes.

In this study, we used an extreme phenotype approach coupled with whole exome sequencing (WES) to discover low-frequency variants associated with severe neuropathy. We selected for WES 8 paclitaxel-treated patients with a long lasting severe neuropathy and with treatment modifications due to neuropathy. Analysis of key genes involved in paclitaxel pharmacokinetics unveiled two *CYP3A4* variants in different patients: a novel and a rare variant (*CYP3A4*25* and *CYP3A4*20*, respectively). Further screening of the full *CYP3A4* coding region in 228 paclitaxel-treated patients revealed five additional carriers of *CYP3A4* rare variants, one not previously described (*CYP3A4*27*). Functional studies showed that the novel variants *CYP3A4*25* and *CYP3A4*27* exhibited decreased CYP3A4 activity.

Statistical analyses confirmed that defective *CYP3A4* variants were overrepresented in paclitaxel-treated patients with severe neuropathy. Moreover, these variants were associated with a 7-fold increased risk of having paclitaxel treatment modifications.

On the whole, we identified for the first time a genetic marker associated with paclitaxel treatment modifications caused by neuropathy. These results support a role for CYP3A4 in paclitaxel-induced neuropathy and provide a basis for paclitaxel treatment individualization.

Personal contribution: I participated in the selection of the 8 patients with severe neuropathy and in the analysis of WES data. I validated the relevant variants by Sanger sequencing and performed *in silico* functional testing for the missense variants. In addition, I performed DHPLC screening of the 13 exons of *CYP3A4* in the validation series and did the statistical analyses (Goodman and Kruskal Gamma test, Fisher exact test and Pearson correlation test). Finally, I contributed to the discussion of the results and I was one of the principal authors drafting the paper.

Clinical Cancer Research

Whole-Exome Sequencing Reveals Defective *CYP3A4* Variants Predictive of Paclitaxel Dose-Limiting Neuropathy

María Apellániz-Ruiz[1], Mi-Young Lee[2], Lara Sánchez-Barroso[1], Gerardo Gutiérrez-Gutiérrez[3], Isabel Calvo[4,5], Laura García-Estévez[5], María Sereno[6], Jesús García-Donás[7], Beatriz Castelo[8], Eva Guerra[9], Luis J. Leandro-García[1], Alberto Cascón[1,10], Inger Johansson[2], Mercedes Robledo[1,10], Magnus Ingelman-Sundberg[2], and Cristina Rodríguez-Antona[1,10]

Abstract

Purpose: Paclitaxel, a widely used chemotherapeutic drug, can cause peripheral neuropathies leading to dose reductions and treatment suspensions and decreasing the quality of life of patients. It has been suggested that genetic variants altering paclitaxel pharmacokinetics increase neuropathy risk, but the major causes of interindividual differences in susceptibility to paclitaxel toxicity remain unexplained. We carried out a whole-exome sequencing (WES) study to identify genetic susceptibility variants associated with paclitaxel neuropathy.

Experimental Design: Blood samples from 8 patients with severe paclitaxel-induced peripheral neuropathy were selected for WES. An independent cohort of 228 cancer patients with complete paclitaxel neuropathy data was used for variant screening by DHPLC and association analysis. HEK293 cells were used for heterologous expression and characterization of two novel CYP3A4 enzymes.

Results: WES revealed 2 patients with rare *CYP3A4* variants, a premature stop codon (*CYP3A4*20* allele) and a novel missense variant (*CYP3A4*25*, p.P389S) causing reduced enzyme expression. Screening for *CYP3A4* variants in the independent cohort revealed three additional *CYP3A4*20* carriers, and two patients with missense variants exhibiting diminished enzyme activity (*CYP3A4*8* and the novel *CYP3A4*27* allele, p.L475V). Relative to *CYP3A4* wild-type patients, those carrying *CYP3A4* defective variants had more severe neuropathy (2- and 1.3-fold higher risk of neuropathy for loss-of-function and missense variants, respectively, $P = 0.045$) and higher probability of neuropathy-induced paclitaxel treatment modifications (7- and 3-fold higher risk for loss-of-function and missense variants, respectively, $P = 5.9 \times 10^{-5}$).

Conclusion: This is the first description of a genetic marker associated with paclitaxel treatment modifications caused by neuropathy. *CYP3A4* defective variants may provide a basis for paclitaxel treatment individualization. *Clin Cancer Res; 21(2); 1–7.* ©*2014 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

M. Apellániz-Ruiz and M.-Y. Lee contributed equally to this article.

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Introduction

Paclitaxel is an antimicrotubular agent widely used for the treatment of many solid tumors. Peripheral neuropathy is the major toxicity limiting the clinical utility of this drug (1, 2). The degree of neuropathy is highly variable among patients, and while some remain asymptomatic throughout treatment, those with severe neuropathy can require paclitaxel dose reductions and treatment suspension, and therefore receive potentially suboptimal treatment. The most severe cases sustain long-term damage to the peripheral nerves, substantially reducing their quality of life (3).

Paclitaxel-induced neuropathy is dose dependent, and there are various clinical conditions that have been suggested as risk factors, such as diabetes mellitus, chronic liver disease, alcoholism, and previous neuropathies (4). Genetic variation has also been suggested as a factor influencing neuropathy risk, based on both genome-wide association studies (5, 6) and candidate gene approaches focused on paclitaxel pharmacokinetics (7). A correlation between the severity of the neuropathy and paclitaxel levels in plasma has been described (8, 9). In fact, it has been shown that common polymorphisms in the two genes encoding paclitaxel metabolizing enzymes in the liver, *CYP2C8* and *CYP3A4*, are associated with a moderately increased risk of developing

Translational Relevance

Paclitaxel is a cytotoxic agent widely used for the treatment of many cancers. Treatment with this drug frequently results in peripheral sensory neuropathy that can seriously affect patients' quality of life. We and others have shown that variant alleles moderately decreasing the expression of genes involved in paclitaxel metabolism (i.e., *CYP2C8*3* or *CYP3A4*22*) are associated with paclitaxel-induced neuropathy. The identification of predictive genetic markers for paclitaxel-induced dose-limiting neuropathy could lead to individualized risk assessment, facilitating treatment decision-making and therefore being of great clinical value. In this study, by whole-exome sequencing of severe paclitaxel-induced peripheral neuropathy patients, we confirm the earlier described implication of CYP3A4 in paclitaxel-induced neuropathy and find an association of *CYP3A4* defective variants with paclitaxel treatment modifications. This study emphasizes the need to screen for rare genetic variants in selected cohorts of patients and may provide a basis for paclitaxel treatment individualization.

neuropathy during paclitaxel treatment (*CYP2C8*3* and *CYP3A4*22* alleles) (8, 10, 11). The identification of predictive genetic markers for paclitaxel-induced dose-limiting neuropathy could lead to individualized risk assessment, facilitating treatment decision-making and therefore being of great clinical value.

The newly developed whole-exome sequencing (WES) technology facilitates the identification of mutations and rare variants in exons and exon/intron boundaries that may potentially be implicated in disease and in extreme phenotypes (12–16). Thus, WES could be applied to unveil novel high-impact alleles of importance for interindividual variation in drug metabolism and adverse drug effects. In this study, we identified a loss-of-function allele and a novel missense variant in the *CYP3A4* gene among 8 patients with severe paclitaxel-induced neuropathy. Further screening for *CYP3A4* variants in an independent patient cohort revealed additional loss-of-function and missense allele carriers. Patients with *CYP3A4* defective variants had higher risk of neuropathy and a large increased risk of paclitaxel dose reductions or treatment suspensions. These results highlight the fact that genetic variants that are rare in the general population might be more prevalent in patient groups developing adverse drug reactions and indeed constitute pharmacogenomic biomarkers of value for individualized therapy.

Patients and Methods

Materials used

Dibenzylfluorescein (DBF), fluorescein, paclitaxel, 3ρ-hydroxypaclitaxel (3OH-P), $NADP^+$, glucose-6-phosphate, and yeast glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (Sigma-Aldrich Sweden AB). Lipofectamine LTX/PLUS and cell medium were purchased from Invitrogen (Life Technologies Europe BV). All solvents for high-performance liquid chromatography (HPLC) assay were obtained from Merck KGaA.

Patients

Blood samples from 8 patients with breast cancer with severe paclitaxel-induced neuropathy were collected at Spanish Hospitals for this study. All 8 patients had developed grade 3 sensory neuropathy (NCI-CTC v4; http://www.eortc.be/services/doc/ctc/) during weekly paclitaxel treatment at a cumulative dose $\leq$800 mg/m^2. Furthermore, the neuropathy resulted in treatment suspensions or paclitaxel dose reductions and continued with at least grade 2 intensity for more than 18 months after termination of paclitaxel treatment (Table 1). Other causes of neuropathy, such as diabetes mellitus, alcoholism, hepatic diseases, AIDS, and previous neuropathies, were ruled out. DNA from an independent cohort of 228 patients with breast and ovarian cancer treated with paclitaxel and recruited in different Spanish hospitals from Madrid, starting on January 2011, was available for genetic analysis. The primary objective of this series was to study paclitaxel-induced peripheral neuropathy, for this reason each patient had a complete neuropathy assessment. For each patient, the following information was available: demographics, tumor characteristics, maximum sensory neuropathy grade during paclitaxel treatment, neuropathy evolution once paclitaxel treatment was ceased, cumulative dose of paclitaxel, and paclitaxel dose reductions and suspensions and their causes (Supplementary Table S1). All patients with cancer were older than 18 years of age, had documented histologic cancer neoplasia, a life expectancy of $\geq$12 weeks and ECOG performance status $\leq$2, adequate bone marrow, renal and hepatic function and no previous history of neuropathy, and had taken some form of contraception.

Table 1. Characteristics of the 8 patients included in WES

Characteristic	N[a]
Age (y)	
Median (min–max)	57 (39–79)
Tumor stage	
I	3
II	3
III	1
IV	1
First-line chemotherapy treatment	
FEC+T[b]	5
FEC+T+H[c]	1
FEC75+T80[d]	1
T+H[e]	1
Chemotherapy cycles	
Min–max	5–8
Paclitaxel dose at grade 3 sensory neuropathy (mg/m^2)	
Median (min–max)	750 (400–800)
Duration of sensory neuropathy grade 2–3[f] (months)	
Median (min–max)	38 (19–66)
Paclitaxel treatment modifications due to neuropathy[g]	
Treatment suspension	4
Dose reduction	4

[a]Unless otherwise indicated.
[b]FEC+T: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2, and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2, every 7 days.
[c]FEC+T+H: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2, and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2 plus herceptin (6 mg/kg loading dose; 2 mg/kg subsequent doses), every 7 days.
[d]FEC75+T80: 5-fluorouracil 600 mg/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 80 mg/m^2, every 7 days.
[e]T+H: paclitaxel 80 mg/m^2 plus herceptin (4 mg/kg loading dose; 2 mg/kg subsequent doses), every 7 days.
[f]Duration of grade 2 or 3 sensorial neuropathy after finishing paclitaxel treatment.
[g]When in the same patient paclitaxel dose was reduced and later treatment was suspended, the patient is included in the table as "treatment suspension".

To ensure homogeneity in neuropathy grading across the different collaborating centers, a qualified nurse (L. Sánchez) trained by a neurologist (G. Gutiérrez-Gutiérrez) interviewed by telephone all patients included in the study in a systematic manner to determine severity of symptoms and impairment in activities of daily living (e.g. extension and intensity of paresthesia, sensitivity, strength in hands and feet) and evaluated the neuropathy grade according to NCI-CTC v4. The recruitment of patients and collection of samples was approved by local internal ethical review committees and all patients gave written informed consent to participate in the study.

WES

WES of DNA samples from the 8 patients with extreme sensory neuropathy (Table 1) was carried out at the National Centre for Genomic Analysis (CNAG). DNA was isolated from peripheral blood using the FlexiGene DNA Kit (Qiagen) and quality control was performed according to electrophoresis and spectrophotometric measurements. The Covaris S2 System (Covaris) was used for DNA fragmentation and exome capture was performed using the SureSelect XT HumanAllExon 50Mb kit (Agilent Technologies). Library size and concentration was determined using Bioanalyzer 2100 (Agilent Technologies). Exome sequencing at a mean coverage >50× was performed using 75-bp paired-end technology in a HiSeq2000 (Illumina). Real-time image analysis and base calling was performed using Illumina's Real Time Analysis software version 1.6 using standard parameters. The GEM (http://algorithms.cnag.cat/wiki/The_GEM_library) and BFAST (17) programs were used to align the reads against the whole human genome (hg19 assembly). To identify single-nucleotide variants (SNV) and insertion-deletions (indels), the SAMtools program was used (http://samtools.sourceforge.net). Variants were filtered to rule out those in genome regions with low mappability, those with a strand bias P value < 0.001 in at least one sample and those with low depth read (<15×), the alternative allele present in <20% of reads, and/or the alternative allele present only in forward or reverse reads.

***CYP3A4* variant detection**

The full *CYP3A4* coding region was amplified in the prospective cohort by PCR using specific primers (Supplementary Table S2). Screening for *CYP3A4* variants was performed using denaturing high-performance liquid chromatography (DHPLC), in the DNA WAVE system 4500 HT (Transgenomic), equipped with a DNASep column (Transgenomic).

Sequencing of PCR products was performed on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems).

Genotyping was performed on 15 ng of genomic DNA using the KASPar SNP Genotyping System (Kbiosciences). All assays included DNA samples with known genotypes and negative controls. The Sequence Detection System ABI PRISM 7900HT (Applied Biosystems) was used to determine fluorescence and for allele assignment.

***CYP3A4* expression vectors and heterologous expression**

The coding region of CYP3A4.1 cDNA (NM_017460.5) was cloned into pCMV5 at the *Xba*I and *Kpn*I restriction enzyme sites, to generate pCYP3A4.1 plasmid. To introduce c.1165C>T and c.1423C>G variants, we used the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's instructions. The correct sequence of *CYP3A4* variant plasmids (pCYP3A4-P389S and pCYP3A4-L475V) was confirmed by Sanger sequencing.

HEK293 cells were cultured in DMEM with 10% fetal bovine serum and 100 U/mL penicillin–streptomycin. Cells were transfected with pCYP3A4.1, pCYP3A4-P389S, pCYP3A4-L475V, or empty pCMV5, together with human cytochrome b5a (pCL-cytb5) using Lipofectamine LTX/PLUS, following the manufacturer's guidelines.

Western blot analysis

Cells were solubilized in RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics). Cell lysates or microsomes, prepared as previously described (18) and containing an equal amount of total protein, were separated using 15% SDS-polyacrylamide gel (18). Membranes were probed with primary antibodies against CYP3A4 (α-hCYP3A4, 1:1,000; ref. 19), human cytochrome b5a (α-cytb5a, 1:1,000; Santa Cruz Biotechnology) and, as loading control, the housekeeping escort chaperone ERp29 (α-ERp29, 1:1,000; ref. 20). The amount of expressed CYP3A4 apoproteins was calculated by densitometric analysis (Image Gauge, v.4.0; Fujifilm) of Western blot bands using a standard calibration curve based on CYP3A4 supersomes (BD Biosciences). To determine protein stability, 48 hours post-transfection HEK293 cells were exposed to 50 μmol/L cycloheximide for 8 hours. The CYP3A4 degradation rate was estimated by immunoblotting and further densitometric analysis of protein bands.

Determination of CYP3A4 enzyme activity

All incubations were conducted as previously described (21, 22) with minor modifications. Briefly, microsomal fractions corresponding to 160 μg of protein were mixed with different concentrations of dibenzylfluorescein in 50 mmol/L potassium phosphate buffer (pH 7.4) and the reaction was initiated by adding a prewarmed NADPH-regenerating system. Fluorescein formation was proportional to incubation time and concentration of microsomes. After 60 minutes, formation of metabolites was measured in a SPECTRAmax Gemini microplate spectrofluorometer (Molecular Devices) and results were analyzed using SoftMax Pro5 software.

Statistical analysis

Michaelis–Menten constants K_m, V_{max}, and intrinsic clearance ($CL_{int} = V_{max}/K_m$) were calculated by nonlinear regression analysis using GraphPad and statistical significance was assessed using a paired t test. Association between neuropathy grade (ranked 0 to 3) and specific genetic groups was assessed using the Goodman and Kruskal Gamma test. Association between treatment modifications (binary variable) and specific genetic groups was assessed using the Fisher exact test. In these analyses, potential confounders were accounted for by stratification using the Mantel–Haenszel test. The Pearson correlation test was used to compare all genetic groups simultaneously (the genetic variable ranked in three groups: wild-type, 0; missense variants, 1; loss-of-function variants, 2) with neuropathy grade and treatment modifications. The analysis SPSS software package v.19 was used for all statistical analyses. P values less than 0.05 were considered statistically significant.

Results

Detection of *CYP3A4*20* and *CYP3A4* c.1165C>T (p.P389S) variants by WES in 2 patients with extreme paclitaxel neuropathy

We first screened for loss-of-function and missense variants in critical genes involved in paclitaxel pharmacokinetics (*CYP3A4*, *CYP2C8*, *ABCB1*, and *SLCO1B3*) in 8 patients with extreme sensory neuropathy, and identified two high-impact variants in *CYP3A4* (Supplementary Table S3). One was the *CYP3A4*20* allele, a rare deleterious indel causing a frameshift and premature stop codon (c.1461_1462insA, p.P488Tfs*494). The other was a missense variant (c.1165C>T, p.P389S) located in the highly conserved CYP β-helix 4; it had not been reported previously, and was given the name *CYP3A4*25* by the CYP allele nomenclature committee (www.cypalleles.ki.se). The patients with these variants were women treated with adjuvant FEC+T for breast cancer that upon paclitaxel treatment developed grade 3 neuropathy with loss of sensitivity in hands and feet, dysesthesia and clumsiness, and walking problems. The *CYP3A4*20* carrier had two paclitaxel dose reductions due to the neuropathy and more than 40 months after paclitaxel treatment the symptoms persisted with improvement to grade 2. The *CYP3A4*25* carrier had suspension of paclitaxel treatment after cycle 6 due to the neuropathy and 19 months after paclitaxel treatment symptoms had decreased to grade 2 neuropathy; 25 months after paclitaxel treatment the patient did not report neuropathy symptoms. Sanger sequencing confirmed the presence of both variants in heterozygosity (Fig. 1).

In the *CYP2C8* gene, encoding the other CYP enzyme metabolizing paclitaxel, and in *SLCO1B3* and *ABCB1*, encoding the uptake and efflux paclitaxel transporters, respectively, we only detected previously described missense variants, none of which were predicted to affect protein function. In addition, with the exception of *CYP3A4*, the frequency of most variants in the patients with extreme neuropathy was similar to that reported in the general population (Supplementary Table S3 shows coding polymorphisms and the regulatory intronic *CYP3A4*22*). Thus, we selected the *CYP3A4* gene for further study.

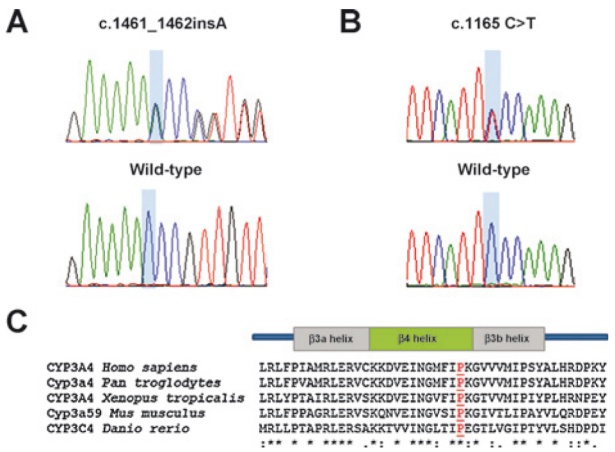

Figure 1.
Coding variants identified by WES in *CYP3A4*. Sanger sequencing confirming the presence of (A) *CYP3A4*20* (c.1461_1462insA) and (B) *CYP3A4*25* (c.1165C>T) variant alleles. The blue-highlighted regions indicate the position of the nucleotide change. C, segment of CYP3A4 protein sequence alignment and predicted secondary structure. Proline 389, marked in red, is highly conserved across species. *, positions with a single, fully conserved residue; ":," amino acids with very similar properties; "." amino acids with mildly similar properties.

Screening for *CYP3A4* variants in a cohort of paclitaxel-treated patients

To determine whether additional *CYP3A4* coding variants are carried by patients with paclitaxel-induced neuropathy, we examined by DHPLC an independent cohort of 228 patients with cancer treated with the drug (Supplementary Table S1). We detected three additional patients carrying the *CYP3A4*20* allele, another patient with *CYP3A4*8* allele (c.389G>A, p.R130Q), and another one with a novel *CYP3A4* missense variant (c.1423C>G, p.L475V, named *CYP3A4*27*). The characteristics of the patients carrying *CYP3A4* coding variants are shown in Table 2.

Stability and enzymatic activity of CYP3A4.25 and CYP3A4.27

The *CYP3A4*20* allele has been shown to encode a nonfunctional enzyme (23) and the *CYP3A4*8* allele was shown to cause decreased CYP3A4 activity (24), but no functional data exist on the novel missense variants *CYP3A4*25* and *CYP3A4*27*. HEK293 cells transiently expressing CYP3A4-P389S or CYP3A4-L475V (CYP3A4.25 and CYP3A4.27, respectively) showed substantially lower amounts of protein compared with CYP3A4-wild-type (CYP3A4.1; Fig. 2). The level of the CYP3A4.27 protein in the expression system was estimated to be 10% of the corresponding expression of CYP3A4.1. The level of CYP3A4.25 was also relatively low, about 40% of the CYP3A4.1 levels and treatment of the cells with the protein synthesis inhibitor cycloheximide confirmed that the P389S substitution in CYP3A4.25 caused an increased rate of degradation (Supplementary Fig. S1). By analyzing the mRNA levels, it was found that the levels were slightly lower (about 80%–70% of the control) for the mutant variants as compared with CYP3A4.1 in the expression system (data not shown). Analyses of catalytic activities of the variant enzymes using dibenzylfluorescein as a CYP3A4 substrate revealed a similar K_m value for CYP3A4.27 (K_m, 7.4 ± 1.8 μmol/L) as compared with CYP3A4.1, whereas the K_m value for CYP3A4.25 was somewhat higher (K_m, 31.7 ± 2.8 μmol/L). The true V_{max} was difficult to determine because of low expression of the variant proteins (data not shown). We conclude that both CYP3A4.25 and CYP3A4.27 have decreased stability in the expression system used.

CYP3A4 variants are associated with an increased risk of neuropathy and paclitaxel treatment modifications

Thus, in a total of 236 patients, composed of 8 WES-studied patients and 228 patients used for *CYP3A4* screening, 4 carried a loss-of-function variant (*CYP3A4*20*) and three carried rare missense variants giving rise to decreased enzymatic activity (*CYP3A4*25*, *CYP3A4*27* and *CYP3A4*8*; Table 2).

The patients with *CYP3A4* loss-of-function variants and patients with missense variants showed a 2- and 1.3-fold increased risk of grade 3 neuropathy, respectively, when compared with wild-type *CYP3A4* patients (Fig. 3A). The neuropathy grade was significantly different between patients with loss-of-function variants and wild-type homozygotes (P = 0.042), and including missense variants in the analysis only minimally changed the P value (P = 0.045). Furthermore, 14% of patients with paclitaxel dose reductions or treatment suspensions due to neuropathy carried *CYP3A4* variants, and a 7- and

Table 2. Characteristics of patients with *CYP3A4* coding variants

Patient	*CYP3A4* variant allele effect	*CYP3A4* genotype	Detection technique[a]	Neuropathy grade[b]	Total cycles (*n*)	Treatment modifications[c]	Time with neuropathy (mo)[d]
11S1213	Loss-of-function	*1/ *20	WES	3	8	Red (at cycles 4 & 8)	(40)
11S919		*1/ *20	DHPLC	3	8	Red (at cycle 4)	(9)
13S812		*1/ *20	DHPLC	3	12	Red (at cycle 9)	(10)
13S120		*1/ *20	DHPLC	3	11	No	16
11S872	Decr. activity	*1/ *25	WES	3	6	Susp (after cycle 6)	19
12S513		*1/ *27	DHPLC	3	8	No	(19)
11S918		*1/ *8	DHPLC	1	12	No	6

[a]Confirmation of variants was performed by Sanger sequencing.
[b]Maximum sensory neuropathy grade during paclitaxel treatment (NCI-CTC v4).
[c]Modifications due to neuropathy. Red, reduction of the paclitaxel dose; Susp, suspension of paclitaxel treatment.
[d]Duration of neuropathy after finishing paclitaxel treatment. For cases for which the neuropathy was ongoing or further evaluation could not be performed because the patient was lost, the duration is written in parentheses.

3-fold increased risk of treatment changes was observed in patients with loss-of-function and missense variants, respectively, when compared with wild-type *CYP3A4* patients (Fig. 3B). This increased risk of treatment modifications for patients with genetically decreased *CYP3A4* activity was statistically significant ($P = 5.8\times10^{-3}$ for loss-of-function variants, $P = 5.9\times10^{-5}$ when missense variants were included in the analysis). When all paclitaxel treatment modifications (i.e., including those due to reasons other than neuropathy) were considered, *CYP3A4* variants were still associated with an increased risk of dose changes ($P = 7.7 \times 10^{-3}$).

Tumor type was not associated with the neuropathy, but conditions considered to be neuropathy risk factors (diabetes, high alcohol intake, restless-legs-syndrome) were significantly associated with neuropathy grade (Supplementary Fig. S2A). Cumulative paclitaxel dose was associated with treatment modifications, as expected, as these result in lower cumulative doses (Supplementary Fig. S2D). Concerning *CYP3A4*22* allele, we found a trend toward higher treatment modifications in carriers of this variant ($P = 0.066$); however, no statistically significant differences were obtained for neuropathy grade and treatment modifications due to neuropathy (Supplementary Fig. S3). Accounting for neuropathy risk factors, cumulative paclitaxel dose or *CYP3A4*22* allele did not substantially change the association observed for *CYP3A4* defective variants.

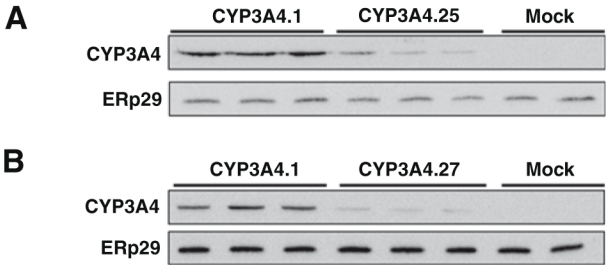

Figure 2.
Expression of CYP3A4.25 and CYP3A4.27. Lysates from transfected HEK293 cells were immunoblotted with antibodies for CYP3A4 and ERp29, the latter as a loading control. A representative image is shown for (A) CYP3A4.25 and (B) CYP3A4.27, with samples loaded in triplicates (duplicates for mock transfected cells). Densitometric analysis estimated that CYP3A4 protein expression was 40% and 10% of the corresponding level of CYP3A4.1, for CYP3A4.25 and CYP3A4.27, respectively.

Discussion

Paclitaxel peripheral neuropathy affects a large number of patients and can lead to treatment modifications (25). Most patients recover from the neuropathy, but long-term nerve damage can also occur, compromising the quality of life of these patients. The extent of paclitaxel exposure is associated with the severity of the neuropathy (8, 9), and paclitaxel elimination is mediated by CYP2C8, CYP3A4, OATP1B3, and P-glycoprotein (7, 26, 27). Thus, alterations in the activity of these proteins could decrease drug elimination and consequently increase toxicity risk (e.g., *CYP2C8*3*, refs. 10, 11; *CYP3A4*22*, refs. 8, 28–30; rs1045642 in *ABCB1*, ref. 31).

The selection of phenotypic outliers has been shown to be an effective strategy to identify genetic variants associated with diseases and drug outcomes (13, 14, 23, 32), and when combined with massive parallel DNA sequencing (33) it is a powerful method to detect low-frequency susceptibility variants (16, 34, 35). Consequently, we designed a study to identify genetic variants in patients with severe paclitaxel-induced neuropathy. Chemotherapy-induced neurotoxicity studies are challenging due to subjectivity of the common toxicity scales and the difficult application of more accurate neuropathy scales across multiple centers (36). In this study, neuropathy was assessed in a systematic manner in all collaborating centers, and for the identification of extreme-phenotype patients, not only the severity of symptoms during treatment, but also modifications of treatment regimen and long-lasting disabilities were taken into account.

WES revealed two rare high-impact variants in *CYP3A4* (*CYP3A4*20* and *CYP3A4*25*). *CYP3A4*20* is an indel leading to a premature stop codon previously described in one individual with impaired elimination of CYP3A4 substrates (23) and the novel CYP3A4.25 (P389S) protein had decreased stability and reduced amounts of apoprotein in the HEK293 expression system (Fig. 2A and Supplementary Fig. S1). In an independent cohort we found three more carriers of *CYP3A4*20*, and two carriers of missense variants, CYP3A4.8, described to have diminished activity (24), and CYP3A4.27, here found to be less expressed in comparison with CYP3A4.1 (Fig. 2B). In total, 3% of the Spanish patients carried *CYP3A4* defective variants, suggesting that these could explain part of CYP3A4 variability. Although *CYP3A4*20* allele is present in Spain it has a low frequency in most European, Asian, and African populations (37) however, alternative *CYP3A4* defective variants might be relevant in other populations. In this

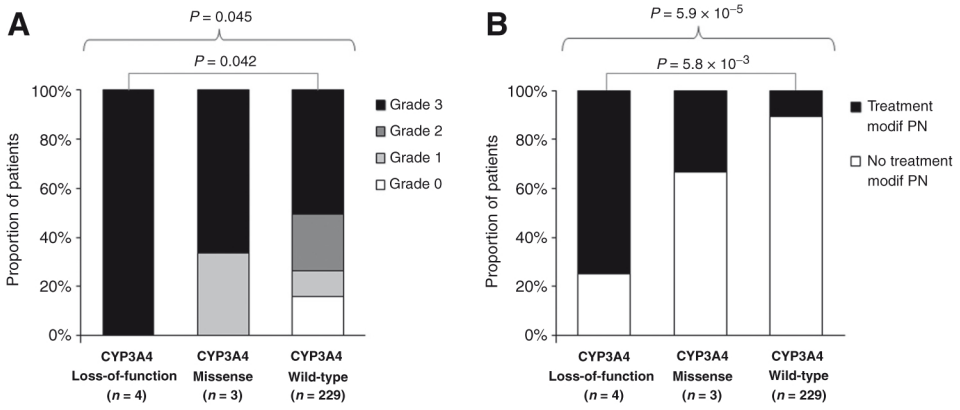

Figure 3.

CYP3A4 defective variants confer an increased risk of paclitaxel-induced neuropathy and treatment modifications. A, neuropathy grade was compared among patients with different CYP3A4 activity. All 4 patients with loss-of-function variants (100%), 2 of 3 patients (67%) with missense variants, and 116 of 229 patients (51%) with wild-type *CYP3A4* had grade 3 sensory neuropathy. B, treatment modifications due to neuropathy were compared among patients with different *CYP3A4* activity. Three of 4 patients (75%) with loss-of-function variants, 1 of 3 patients (33%) with missense variants, and 25 of 229 patients (11%) with wild-type *CYP3A4* had treatment modifications. As described in Patients and Methods, the Goodman and Kruskal gamma test and Fisher exact test were used to assess association with neuropathy grade ($\gamma = 1$) and treatment modifications. To perform an analysis including simultaneously all variants categorized according to CYP3A4 activity (loss-of-function, missense, and wild-type), Pearson correlation test was used. Treatment modif PN, treatment modifications due to peripheral neuropathy.

respect, 3% of European Americans and 2% of African Americans seem to carry potentially defective *CYP3A4* allele variants (loss-of-function or missense variants likely damaging according to Polyphen; see Exome Variant Server database).

Patients carrying loss-of-function *CYP3A4* variants had a significantly higher risk of neuropathy and paclitaxel treatment modifications, when compared with wild-type *CYP3A4* patients ($P = 0.042$ and $P = 5.8 \times 10^{-3}$, respectively; Fig. 3), and carriers of missense variants showed an intermediate phenotype, concordant with a decreased but not abolished CYP3A4 activity. For *CYP3A4*22* allele, we only detected a trend toward increased treatment modifications, which might reflect a lower effect of this variant on paclitaxel metabolism and/ or a low statistical power due to the small number of *CYP3A4*22* carriers (Supplementary Fig. S3). Four of the 29 patients with paclitaxel treatment modifications due to neuropathy carried *CYP3A4* defective variants (missense or loss-of-function), indicating that genetic testing of *CYP3A4* before treatment, would have a very high specificity (99%) but poor sensitivity (14%). If pathologic risk factors were also taken into consideration, the sensitivity would increase, and an estimated 27% of patients carrying *CYP3A4* defective variants or with preexisting conditions associated with neuropathy, would require treatment modification due to severe neuropathy upon paclitaxel chemotherapy. Paclitaxel-induced neuropathy is a multifactorial and polygenic trait, and additional genetic variants, some yet to be identified, will further improve the predictive power of genetic testing. In this regard, Supplementary Table S4 shows genetic variants that have been described as moderate risk factors for paclitaxel-induced neuropathy (*CYP3A4*22*, *CYP2C8*3*, *EPHA5*-rs7349683, and *XKR4*-rs4737264, the latter two identified in a meta-analysis of genome-wide association studies; refs. 5, 6). Thus, the identification of genetic variants and physiopathologic risk factors predictive of paclitaxel-induced neuropathy may provide a basis on which to individualize this treatment. It is important to highlight that previous reports have only identified markers associated to neuropathy grade, but not with neuropathies resulting in treatment modifications. In fact, this is the first study describing a marker associated with paclitaxel dose-limiting neuropathy.

In summary, in this study we found an overrepresentation of defective *CYP3A4* variants in patients with paclitaxel treatment modifications and in those with high-grade paclitaxel-induced neuropathy. This supports and confirms the earlier described implication of CYP3A4 in paclitaxel-induced neuropathy. These results emphasize the need to screen for rare genetic variants in selected cohorts of patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Characteristics of the 228 prospectively recruited breast and ovarian cancer patients treated with paclitaxel and used for the detection of *CYP3A4* coding variants.

Characteristics	**N (%)***
Age (years)	
Median (min-max)	51 (30-81)
Tumor type	
Breast	200 (88%)
Ovary	28 (12%)
Type of paclitaxel treatment	
First line	221 (97%)
Second line[a]	7 (3%)
Paclitaxel chemotherapy treatment[b]	
FEC+T[c]	111 (49%)
AC+T[d]	35 (15%)
T+FEC[e]	29 (13%)
C+T[f]	27 (12%)
Others	26 (11%)
Number of paclitaxel cycles	
(min-max)	8 (3-12)
Maximum sensory neuropathy grade[g]	
G0	36 (16%)
G1	25 (11%)
G2	53 (23%)
G3	114 (50%)
Treatment modifications due to neuropathy[h]	
Dose reduction	5 (2%)
Treatment suspension	16 (7%)
All treatment modifications	
Dose reduction	21 (9%)
Treatment suspension	21 (9%)

*Unless otherwise stated

[a] Patients with second line paclitaxel treatment and no previous cytotoxic drugs in first line therapy.

[b] Some patients receiving chemotherapeutic drugs in combination with targeted therapy (bevacizumab, trastuzumab, denosumab or pertuzumab) are included in the table according to the chemotherapy agents received.

[c] FEC+T: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2, every 7 days.

[d] AC+T: doxorubicin 60mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 80mg/m^2, every 7 days.

[e] T+FEC: paclitaxel 80 mg/m^2, every 7 days, followed by 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days.

[f] C+T: carboplatin AUC5-6 and paclitaxel 175mg/m^2, every 21 days.

[g] NCI-CTC v4.

[h] When in the same patient's paclitaxel dose was reduced and then later suspended, the patient is included in the table as "treatment suspension".

Supplementary Table S2. Primers used for *CYP3A4* sequencing.

Exon	Primer sequence	Fragment size (bp)
E1 FW	5’ TTCTTTGCCAACTTCCAAGG3’	378
E1 RV	5’AAGGGAAAGAGAGGCCTGA3’	
E2 FW	5’TCGTTCTCTTGAGCATTCCA3’	247
E2 RV	5’AAGCTGCTCTTGGCAATCAT3’	
E3 FW	5’GGCTTCCACTGTTTTCATCC3’	221
E3 RV	5’TTGGGCTGAGACTGTCCTCT3’	
E4 FW	5’TTGGGCTCCAGCTGTAGAAT3’	582
E4 RV	5’CCACATGGAGACAGAGTGGA3’	
E5 FW	5’ CCATGGAGACCTCCACAACT3’	271
E5 RV	5’ CAGTGGACTACCCCTTGGAA3’	
E6 FW	5’TCACTTACTGCTCCATGCTG3’	546
E6 RV	5’TGCCAACCAACAGATACCAA3’	
E7 FW	5’ATGTGGGTTTCCTGTTGCAT3’	374
E7 RV	5’TGATGGTCACACATATCTTCAAA3’	
E8 FW	5’TGGCTTCCAGTTGAGAACCT3’	379
E8 RV	5’GAGCAGTCTTCATGTTAAAAGCA3’	
E9 FW	5’TCAGGAGCCACTTTCTGTCA3’	283
E9 RV	5’GCCTGCATGCCTCTAGAAAG3’	
E10 FW	5’AGGGATTTGAGGGCTTCACT3’	334
E10 RV	5’TTCTCCTGGGAAGTGGTGAG3’	
E11 FW	5’AAATGCTTCGATCCTTTACCA3’	415
E11 RV	5’GGCAGAATATGCTTGAACCAG3’	

Exon	Primer sequence	Fragment size (bp)
E12 FW	5’GGGTGGCCCCTAAGTAAGAA3’	392
E12 RV	5’TCACAGATGGGCCTAATTGA3’	
E13 FW	5’GTCCCCTCAACACTGAAGGA3’	202
E13 RV	5’GGAAAATTCAGGCTCCACTT3’	

Supplementary Table S3. Loss-of-function, missense and known regulatory variants in genes mediating paclitaxel pharmacokinetics in the eight patients with extreme paclitaxel-induced neuropathy studied by WES.

Gene	Variant ID/ common name	Type of variant	Protein change	No variant carriers (het/hom)	MAF[a] (%)		Predicted effect[b]
					Obs	Eur	
CYP3A4	c.1165C>T/ novel (*25)	Missense	P389S	1/0	6	**ND**[c]	**Altered function (4)**
	rs67666821/ *20	**Frameshift**	**P488Tfs*494**	1/0	6	**0.09**	-[d]
	rs35599367/ *22	Intronic	-	1/0	6	5	-[d]
CYP2C8	rs11572080/ *3	Missense	R139K	2/0	12	12	Neutral (4)
	rs10509681/ *3	Missense	K399R	2/0	12	12	Neutral (4)
	rs1058930/ *4	Missense	I264M	1/0	6	6	Neutral (2)
ABCB1	rs9282564	Missense	N21D	1/0	6	10	Neutral (4)
	rs60419673	Missense	N183S	1/0	6	0.2	Neutral (3)
	rs2032582/ 2677GT	Missense	A893S	5/1	43	43	Neutral (3)
SLCO1B3	rs188817665	Missense	G653E	1/0	6	1	Neutral (3)

[a] Minor allele frequency observed in the eight patients studied by WES (Obs) and in European Americans (Eur) according to Exome Variant Server (ESP2), or 1000 Genomes Project for the intronic rs35599367.

[b] The effect of the missense changes was predicted by using 4 algorithms: PolyPhen (Adzhubei et al., 2010), SIFT (Ng and Henikoff, 2003), MutPred (Li et al., 2009)and SNPs&Go (Calabrese et al., 2009). For MutPred a threshold score of 0.5 was used. The number of algorithms with consistent results is indicated in brackets.

[c] Not described.

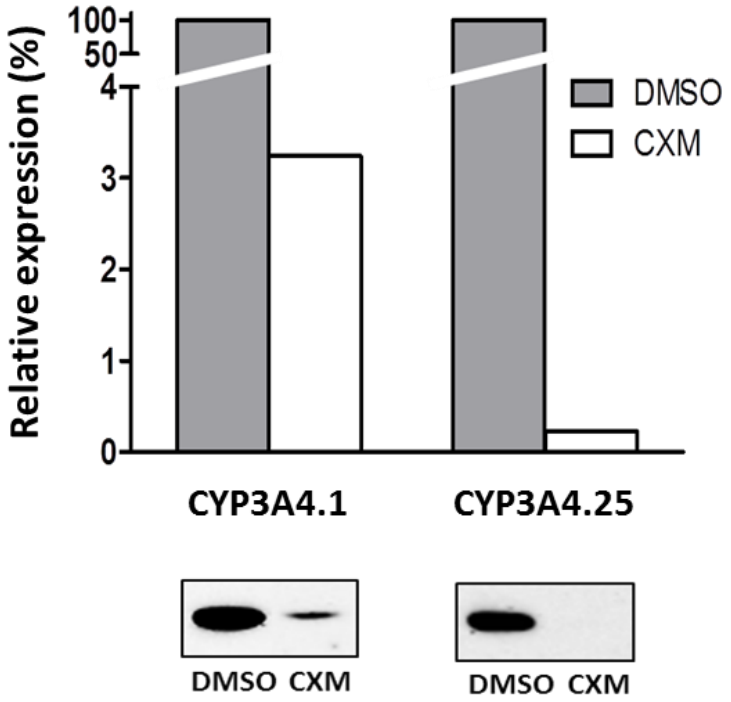
[d] Predictions for frameshift and intronic variants are not performed by the algorithms. However, functional studies have shown that rs67666821 leads to a non-functional truncated protein (Westlind-Johnsson et al., 2006) and that rs35599367 decreases *CYP3A4* expression (Elens et al., 2013a).

Supplementary Table S4. Genotype of variants previously associated with paclitaxel-induced neuropathy, in the patients with defective *CYP3A4* coding variants.

Gene	Variant common name	Variant ID	Nr. of neuropathy risk alleles						
			11S1213	**11S919**	**13S812**	**13S120**	**11S872**	**12S513**	**11S918**
CYP3A4	*CYP3A4*20*	rs67666821 (frameshift)	**1**	**1**	**1**	**1**	0	0	0
	*CYP3A4*25*	novel (P389S)	0	0	0	0	**1**	0	0
	*CYP3A4*27*	novel (L475V)	0	0	0	0	0	**1**	0
	*CYP3A4*8*	rs72552799 (R130Q)	0	0	0	0	0	0	**1**
CYP3A4	*CYP3A4*22*	rs35599367	**1**	0	0	0	0	0	0
CYP2C8	*CYP2C8*3*	rs11572080/ rs10509681	**1**	**1**	0	0	0	0	**1**
EPHA5	-	rs7349683	0	**2**	0	0	**1**	**2**	0
XKR4	-	rs4737264	**1**	**1**	0	0	**1**	0	**1**
***CYP3A4* coding variants**			**Loss-of-function (no activity)**				**Missense (decreased activity)**		
Paclitaxel-induced neuropathy (grade/ dose modif.) [a]			3/ Red (2)	3/ Red	3/ Red	3/ -	3/ Susp	3/ -	1/ -

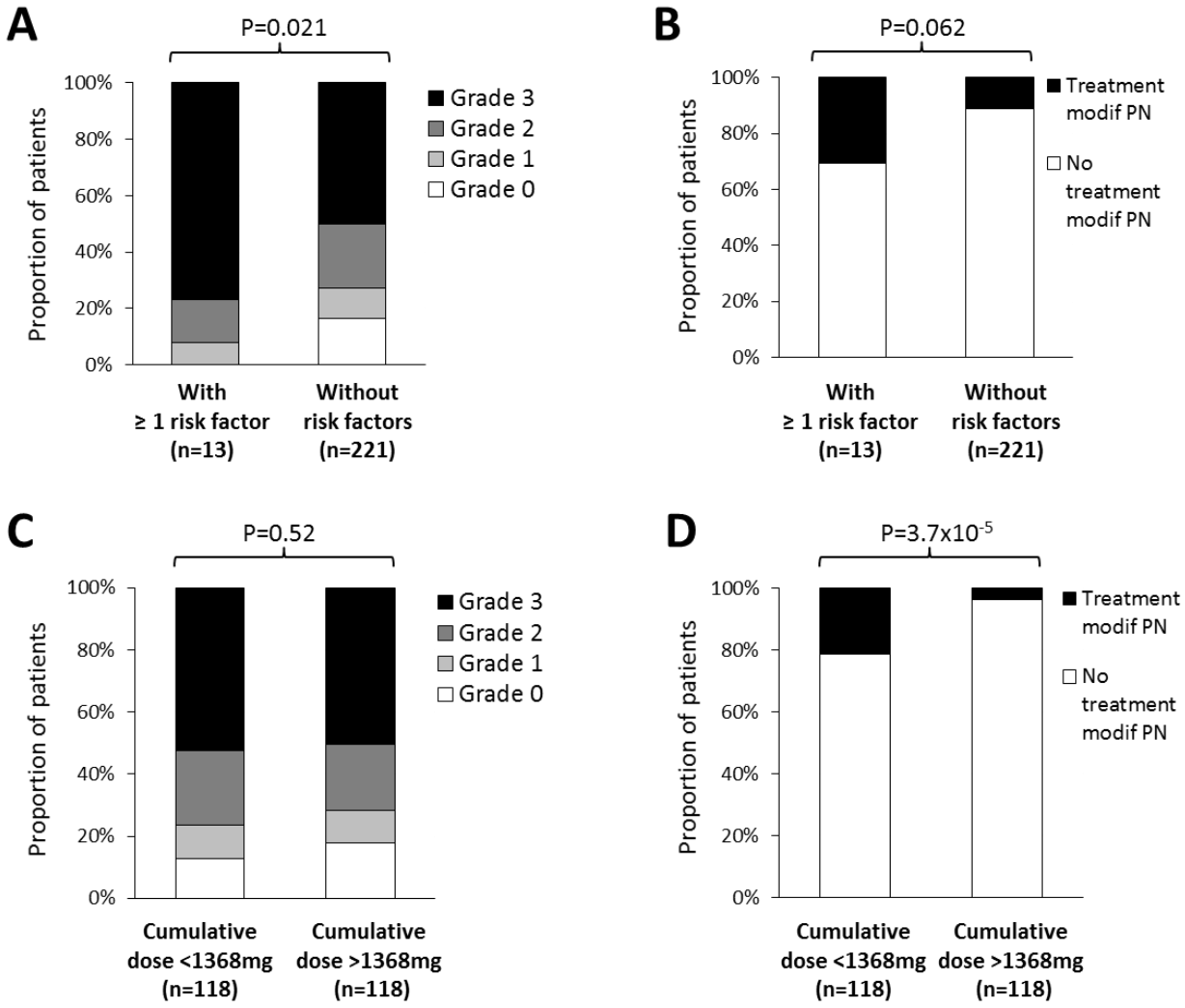
[a] Dose modif, paclitaxel dose modifications; Red, paclitaxel dose reduction due to neuropathy; Susp, paclitaxel treatment suspension due to neuropathy.

Supplementary Figure S1



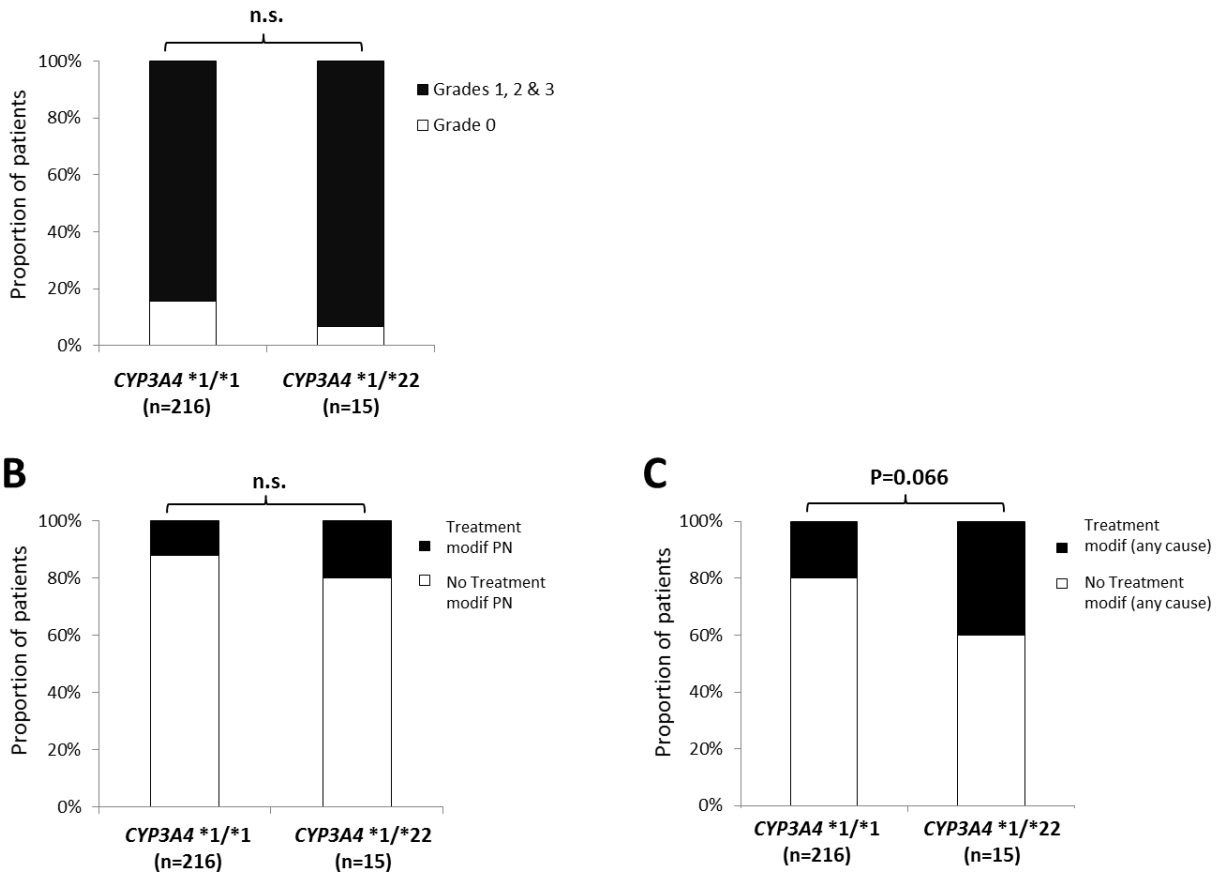
CYP3A4.25 protein stability assessment. Degradation rate of CYP3A4.25 protein compared to CYP3A4.1. 48 hour post-transfection HEK293 cells were exposed to 50 µM cycloheximide (CXM) or vehicle (0.06% DMSO) for 8 hours. Upper panel, relative protein levels estimated by densitometric analysis of western blot bands. Lower panel, western blot results. Each lane was loaded with the cell lysate containing equal amounts of total protein. Membranes were overexposed to visualize the protein bands after CXM treatment.

Supplementary Figure S2



Neuropathy risk conditions confer an increased risk of paclitaxel-induced neuropathy and a trend towards treatment modifications. Neuropathy grade (**A**) and treatment modifications due to neuropathy (**B**), were compared between patients with neuropathy risk conditions (diabetes mellitus, high alcohol intake or restless-legs-syndrome) and patients without risk conditions. Two patients were excluded from the analysis due to lack of information regarding confounding factors. The median cumulative paclitaxel dose was used to group patients according to high or low paclitaxel exposure. Neuropathy grade (**C**) and treatment modifications due to neuropathy (**D**), were compared between patients with different cumulative paclitaxel doses. The Goodman and Kruskal's gamma test and Fisher exact test were used to assess associations with neuropathy grade (γ=1) and treatment modifications, respectively. Treatment modif PN, treatment modifications due to peripheral neuropathy.

Supplementary Figure S3



***CYP3A4*22* association with paclitaxel-induced neuropathy and paclitaxel dose modifications.** Neuropathy grade (**A**), treatment modifications due to neuropathy (**B**) and treatment modifications due to any cause (**C**), were compared between *CYP3A4*22* heterozygous carriers (*CYP3A4*1/*22*) and *CYP3A4* wild type patients (*CYP3A4*1/*1*). Five patients were excluded from the analysis due to missing *CYP3A4*22* genotype. The Goodman and Kruskal's gamma test and Fisher exact test were used to assess associations with neuropathy grade (γ=1) and treatment modifications, respectively. Treatment modif PN, treatment modifications due to peripheral neuropathy. Treatment modif (any cause), treatment modifications due to neuropathy or to any other cause.

ARTICLE 4: High frequency and founder effect of the *CYP3A4*20* loss-of-function allele in the Spanish population classifies CYP3A4 as a polymorphic enzyme.

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Abstract:

Cytochrome P450 3A4 (CYP3A4) is a key drug metabolizing enzyme involved in the biotransformation of more than half of all clinically used drugs. Genetic factors have been estimated to account for a high percentage of the inter-individual variability observed in CYP3A4 expression. Despite these evidences, loss-of-function variants have only been reported as rare events.

One of these rare loss-of-function alleles is *CYP3A4*20*. Interestingly, we found four carriers of this variant among Spanish patients with severe neuropathy. As no data was available regarding *CYP3A4*20* allele origin, we characterized the world distribution and origin of *CYP3A4*20* mutation.

*CYP3A4*20* was genotyped in more than 3750 individuals representing different populations and haplotype analysis was performed using *CYP3A* polymorphisms and microsatellite markers.

We found that *CYP3A4*20* allele was present in 1.2% of the Spanish population (up to 3.8% in specific regions), and that all *CYP3A4*20* carriers shared a common haplotype. These results were compatible with a Spanish founder effect. In addition, it is the first description of a loss-of-function *CYP3A4* allele with a relatively high frequency in a population, classifying CYP3A4 as a polymorphic enzyme.

The key role of CYP3A4 in drug metabolism, together with evidences of increased risk of adverse drug reactions in *CYP3A4*20* carriers, suggest the relevance of implementing *CYP3A4*20* screening in the Spanish population.

Personal contribution: I participated in the design of the study, performed SNP genotyping and microsatellite testing through DNA fragment analysis. I performed haplotype analysis and participated in statistical analyses. Finally, I contributed to the discussion of the results and I was the principal author drafting the paper.

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ORIGINAL ARTICLE

High frequency and founder effect of the *CYP3A4*20* loss-of-function allele in the Spanish population classifies CYP3A4 as a polymorphic enzyme

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Cytochrome P450 3A4 (CYP3A4) is a key drug-metabolizing enzyme. Loss-of-function variants have been reported as rare events, and the first demonstration of a CYP3A4 protein lacking functional activity is caused by *CYP3A4*20* allele. Here we characterized the world distribution and origin of *CYP3A4*20* mutation. *CYP3A4*20* was determined in more than 4000 individuals representing different populations, and haplotype analysis was performed using *CYP3A* polymorphisms and microsatellite markers. *CYP3A4*20* allele was present in 1.2% of the Spanish population (up to 3.8% in specific regions), and all *CYP3A4*20* carriers had a common haplotype. This is compatible with a Spanish founder effect and classifies CYP3A4 as a polymorphic enzyme. This constitutes the first description of a *CYP3A4* loss-of-function variant with high frequency in a population. *CYP3A4*20* results together with the key role of CYP3A4 in drug metabolism support screening for rare *CYP3A4* functional alleles among subjects with adverse drug events in certain populations.

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INTRODUCTION

Cytochromes P450 (CYPs) are the most important drug-metabolizing enzymes, being CYP1, 2 and 3 families responsible for the biotransformation of ~70–80% of all therapeutic compounds.[1,2] CYP 3A4 (CYP3A4) is the most abundant P450 enzyme in the human liver and gastrointestinal tract and it is involved in the biotransformation of more than half of all clinically used drugs.[1–3] There is a high variability in CYP3A4 expression (>100-fold),[1] caused by non-genetic and genetic factors, which contributes to unpredictable drug responses and toxicities. Among environmental factors, drug–drug interactions are one of the most studied causes of variation, but gender, hormonal status and age also influence CYP3A4 expression and activity.[4,5] With respect to genetic factors, twin studies and repeated drug administration approaches have estimated a high degree of heritability in the CYP3A4 interindividual variation.[6–8] In this regard, the Human CYP Allele Nomenclature Database includes 26 different CYP3A4 variant proteins. Three are truncated proteins resulting from rare premature stop codons (*CYP3A4*6, CYP3A4*20* and *CYP3A4*26* alleles),[9,10,11] whereas the rest are low-frequency/rare missense variants, some with reduced enzymatic activity (e.g. *CYP3A4*8, *11, *13, *16* and **17* alleles; http://www.cypalleles.ki.se/). In addition, two noncoding SNPs have been related to altered gene expression (e.g. *CYP3A4*1B* and *CYP3A4*22*).[12,13] On the whole, although several genetic variants that affect CYP3A4 activity have been described, in general these are rare or low-frequency alleles expected to explain only a small fraction of CYP3A4 phenotypic variability.[14]

The *CYP3A4*20* allele, a single base pair (A) insertion causing a frameshift and premature stop codon in the protein (c.1461_1462insA; p.P488Tfs*494), is one of the two *CYP3A4* gene variants in which lack of enzymatic activity has been demonstrated. This allele was found in heterozygosity in an individual of Brazilian descent with a sixfold increased exposure of a drug metabolized by CYP3A4 and low systemic midazolam clearance,[9] and classified as rare, as no *CYP3A4*20* carriers were found in 428 Germans. This variant has not been described again, and there is no data regarding its possible origin and distribution in different populations. However, we found one *CYP3A4*20* carrier upon whole-exome sequencing of Spanish patients with severe toxicity.[15] This finding triggered this study investigating the *CYP3A4*20* allele distribution in Spain and worldwide. We find that 1 in 82 Spanish individuals carries this allele, and haplotype analyses suggest a Spanish founder effect. This is the first description of a loss-of-function *CYP3A4* allele with a relatively high frequency in a population, and demonstrates the polymorphic nature in this gene.

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MATERIALS AND METHODS

DNA from control individuals

DNA isolated from blood samples (FlexiGene DNA Kit; Qiagen, Hilden, Germany) of 1977 Spanish, 450 Portuguese, 478 Italian, 240 Argentinean, 199 Bolivian, 29 Algerian, 95 Libyan, 117 Israeli, 133 Saudi Arabian, 83 Kuwaiti, 186 Pakistani and 108 Chinese controls were collected. All individuals were over 18 years, the collection of samples was approved by local internal ethical review committees and investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Genotyping of SNPs in *CYP3A* genes

In addition to *CYP3A4*20* (rs67666821), the *CYP3A* single-nucleotide polymorphisms (SNPs) *CYP3A4*22* (rs35599367), *CYP3A4*1B* (rs2740574), *CYP3A5*3* (rs776746) and *CYP3A7*2* (rs2257401) alleles were selected for genotyping using the KASPar SNP Genotyping System (LGC Genomics, Herts, UK) with 15 ng of genomic DNA. All assays included DNA control samples with known genotypes and negative controls. The Sequence Detection System ABI PRISM 7900HT (Applied Biosystems, Darmstadt, Germany) was used for fluorescence detection and allele assignment. The accuracy of the genotyping was confirmed by sequencing all *CYP3A4*20* carriers, and a random selection of individuals with different *CYP3A4*22, CYP3A4*1B, CYP3A5*3* and *CYP3A7*2* genotypes. PCR products were purified and Sanger sequencing run on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems).

Microsatellites markers

For haplotype analysis, a panel of four microsatellite markers on chromosome 7q21-22 spanning an interval of 3.2 Mb was used: D7S651, D7S2498, D7S2480 and D7S666 (Supplementary Figure 1). In brief, PCR was carried out using specific primers, and with the forward primers labeled with 6-Fam fluorochrome. The diluted PCR products were mixed with Hi-Di Formamide and LIZ-500 size standard (Applied Biosystems), separated and detected using an ABI Prism 3100 automatic sequencer (Applied Biosystems) and analyzed by Peak Scanner software version 1 (Applied Biosystems).

Haplotype analysis and dating the origin of *CYP3A4*20* allele

Haplotypes were identified using SNPs (*CYP3A4*22*, *CYP3A4*1B*, *CYP3A5*3* and *CYP3A7*2*) and the four microsatellite markers in 20 *CYP3A4*20* carriers (19 Spanish and 1 Portuguese) and in 50 Spanish individuals wild type for this variant, using PHASE software (University of Chicago, USA, http://c4c.uwc4c.com/express_license_technologies/phase).[16]

The mutation origin of *CYP3A4*20* variant was calculated using DMLE+ software version 2.3 developed by Reeve and Rannala[17] (http://dmle.org/). This program uses the Markov Chain Monte Carlo algorithm to allow Bayesian estimation of the mutation age based on: the observed haplotypes in variant carriers and unrelated normal individuals, map distances between markers, the position of the mutation relative to the markers and the estimated population growth rate.

Statistical analysis

Hardy–Weinberg equilibrium was tested for the SNPs genotyped and none significantly deviated from expected values.[18] Fisher's exact test was used to examine the association between *CYP3A4*20* allele frequency and country of origin. *P*-values below 0.05 were considered statistically significant. SPSS version 19 was used for the statistical analysis.

RESULTS

*CYP3A4*20* allele distribution in different populations

The Exome Variant Server (EVS) database (http://evs.gs.washington.edu/EVS/) suggested that the loss-of-function *CYP3A4*20* allele is rare but detectable in some individuals, mainly of European origin, and our finding of one carrier in a Spanish individual led us to carry out a *CYP3A4*20* allele frequency population study in individuals from European, African and Asian descent (Table 1 and Figure 1). As expected from previous data, no *CYP3A4*20* carriers were found in Italians, Argentineans, Bolivians and individuals from different countries in Africa and Asia. However, the *CYP3A4*20* variant was detected in heterozygosity in 24 Spanish individuals and in 1 Portuguese, revealing that 1.2% and 0.2%, respectively, of these populations carried the variant (Table 1). The unexpected high number of *CYP3A4*20* allele carriers in the Spanish individuals was significantly different from the other populations studied (Fisher's exact test $P < 0.0001$).

Characterization of *CYP3A4*20* allele frequency in different Spanish regions

To determine *CYP3A4*20* allele distribution within Spain, we collected the place of birth of 1544 individuals among the 1953 genotyped Spanish controls. When comparing the *CYP3A4*20* allele frequency with the region of birth, we found that the variant had the highest frequency in individuals from Castilla y León,

Table 1. Distribution of *CYP3A4*20* allele in different populations

Country	*Population*	CYP3A4 *wild-type homozygous (no.)*	CYP3A4*20 *heterozygous (no.)*	CYP3A4*20 *carriers (%)*	*Reference*
Spain	European	1953	24	1.21	This study
Portugal		449	1	0.22	This study
Italy		478	0	0	This study
Germany		428	0	0	Westlind-Johnsson *et al.*[9]
Argentina[a]		240	0	0	This study
Bolivia[b]		199	0	0	This study
European Americans		4119	8	0.19	EVS[c]
Libya	African	95	0	0	This study
Algeria		29	0	0	This study
African Americans		2131	1	0.05	EVS[c]
Israel	Asian	117	0	0	This study
Saudi Arabia		133	0	0	This study
Kuwait		83	0	0	This study
Pakistan		186	0	0	This study
China		108	0	0	This study

Abbreviations: CYP3A4, cytochrome P450 3A4; EVS, Exome Variant Server. [a]Classified as an European population because of the high number of Argentinians of European origin. [b]In Bolivian population, the European ancestry is 13–21% and the Native American component 77–86%. [c]Data from EVS (http://evs.gs.washington.edu/EVS/).

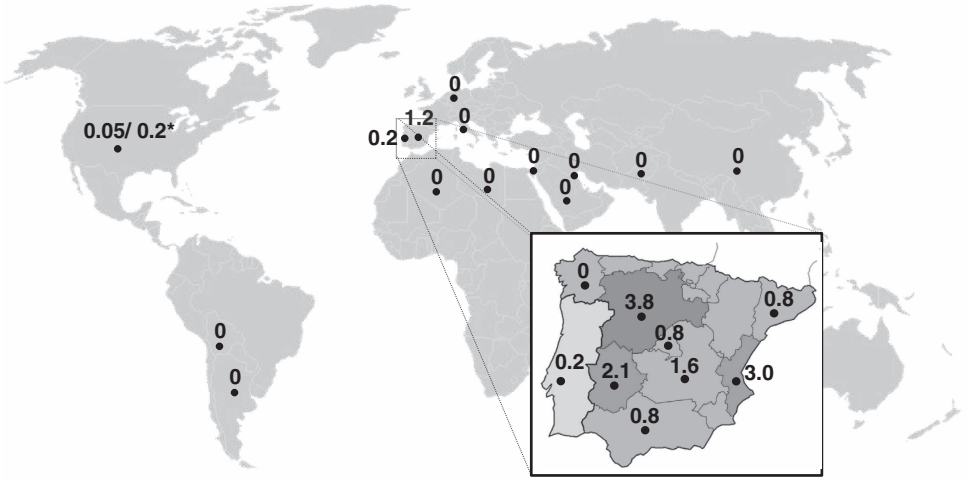

Figure 1. Geographical distribution of *CYP3A4*20* allele. World map showing the percentage of *CYP3A4*20* allele carriers in different populations. The Spanish peninsula is shown in greater detail. Number of carriers and individuals studied are presented in Table 1 and in Supplementary Table 1. *Concerning United States of America, the data corresponds to the EVS, the first number refers to African Americans and the second to European Americans.

D7S666	D7S2480	D7S2498	*CYP3A4 *1B*	*CYP3A4 *22*	*CYP3A4 * 20*	*CYP3A7 * 2*	*CYP3A5 * 3*	D7S651	Nr Haplotypes	
x	282	172	A	C	InsA	C	G	x	20	Indiv *CYP3A4*20* (n=20)
x	282	172	A	C	-	C	G	x	4	
x	286	172	A	C	-	C	G	x	18	
x	270	172	A	C	-	C	G	x	9	
x	288	172	A	C	-	C	G	x	4	
x	272	172	A	C	-	C	G	x	1	
x	280	172	A	C	-	C	G	x	1	
x	284	172	A	C	-	C	G	x	1	
x	290	172	A	C	-	C	G	x	1	
x	282	180	A	C	-	C	G	x	12	
x	286	174	A	C	-	C	G	x	1	
x	270	180	A	C	-	C	G	x	1	
x	284	180	A	C	-	C	G	x	1	
x	290	181	A	C	-	C	G	x	1	
x	286	182	A	C	-	C	G	x	1	
x	286	180	A	C	-	C	G	x	11	
x	290	180	A	C	-	C	G	x	10	
x	288	180	A	C	-	C	G	x	4	
x	288	182	A	C	-	C	G	x	3	
x	290	176	A	C	-	C	G	x	2	
x	286	180	A	T	-	C	G	x	3	
x	270	180	A	T	-	C	G	x	1	
x	288	180	A	T	-	C	G	x	2	
x	270	166	A	C	-	G	G	x	1	
x	270	172	A	C	-	G	G	x	2	
x	270	172	A	C	-	G	A	x	3	
x	288	180	A	C	-	G	A	x	1	
x	288	180	G	C	-	G	A	x	1	

Indiv not *CYP3A4*20* (n=50)

Figure 2. *CYP3A4*20* haplotype analysis. Haplotypes were identified using PHASE in 20 *CYP3A4*20* carriers and 50 Spanish control individuals with 5 SNPs (*CYP3A4*1B, CYP3A4*22*, *CYP3A4*20, CYP3A7*2* and *CYP3A5*3*) and 4 microsatellite markers at 7q (D7S666, D7S2480, D7S2498 and D7S651), spanning an interval of 3.2 Mb. For the 20 *CYP3A4*20* carriers only the haplotype containing the mutation is shown; for the 50 non-carriers the 100 haplotypes are shown. D7S666 and D7S651 showed variability within the *CYP3A4*20* haplotype, this is indicated as "x".

Comunidad Valenciana and Extremadura, where we found one heterozygous every 26, 33 and 48 individuals, respectively, resulting in 3.8%, 3.0% and 2.1% *CYP3A4*20* carriers (Figure 1 and Supplementary Table 1). In other Spanish regions, the proportion of variant carriers ranged from 1.6 to 0.8%, with the exception of Galicia, where no variant carriers were found (Figure 1 and Supplementary Table 1).

*CYP3A4*20* ancestral haplotype

To investigate whether all occurrences of *CYP3A4*20* allele descended from a single ancestral mutation event or arisen independently, we constructed haplotypes with *CYP3A4*20*, four SNPs in *CYP3A* locus and four microsatellite markers (Supplementary Figure 1). Haplotype reconstruction in 20 *CYP3A4*20* allele carriers suggested that all carriers showed a common haplotype (282, 172, A, C, InsA, C, G) that contained this variant and spanned ~700 kb (from microsatellite D7S2480 to SNP *CYP3A5*3*; Figure 2 and Supplementary Figure 2). The *CYP3A4*20* haplotype contained wild-type alleles for *CYP3A4*22*, *CYP3A4*1B* and *CYP3A7*2*, and carried *CYP3A5*3*, the most common variant in Caucasians. In 50 individuals wild type for *CYP3A4*20*, representing the control Spanish population, 27 different haplotypes existed with frequencies ranging from 18 to 1% (Figure 2). Further-

more, 4 out of the 100 chromosomes analyzed were predicted to carry the same haplotype as *CYP3A4*20* but without this mutation (282, 172, A, C, –, C, G). This result suggests a single ancestral allele in which the variant was likely originated.

Age of *CYP3A4*20* variant

The decay of linkage disequilibrium due to recombination can be used to date the age of a mutation. We used the DMLE+ software to estimate the age of *CYP3A4*20* variant using the haplotype data from the 20 *CYP3A4*20* carriers and 50 Spanish controls previously studied. The mutation age was estimated to be 51 generations (95% credible interval of 43–60) using an average growth rate of 0.25. Assuming 20 years for a generation, the age of the variant was estimated to be 1020 years old. For growth rates of 0.15 and 0.35, mutation age was estimated to be 82 and 38 generations, respectively.

DISCUSSION

In contrast to the high polymorphic nature of most drug-metabolizing enzymes, *CYP3A4* gene exhibits little genetic variability. The Human CYP Allele Nomenclature Database (http://www.cypalleles.ki.se/) includes only three loss-of-function *CYP3A4* alleles (*CYP3A4*6, CYP3A4*20* and *CYP3A4*26*) and the EVS database (http://evs.gs.washington.edu/EVS/) suggests that only 0.2% of Americans carry *CYP3A4*-defective variants and 2% missense variants, many of which have unknown functional significance. The *CYP3A4*20* allele, which encodes a truncated protein devoid of catalytic activity,[9] is the most common *CYP3A4*-defective allele in the EVS database with 0.2% and 0.05% of European Americans and African Americans carriers, respectively (i.e. 8 carriers out of 4127 and 1 carrier out of 2132 individuals), whereas it was not detected in 428 German individuals.[9] In the present study, we found that 1.2% of the Spanish population (24 out of 1977 individuals) carry the *CYP3A4*20* allele, compared with 0.2% in Portugal (1 out of 450) and no carriers in Italy, Argentina, Bolivia, Libya, Algeria, Israel, Kuwait, Saudi Arabia, Pakistan and China (Figure 1). On the whole, one in 82 Spanish carried this variant. Within Spain, this figure increased to one *CYP3A4*20* carrier every 26 individuals in Castilla y León, one in 33 in Comunidad Valenciana one in 48 individuals in Extremadura. These results constitute the first proof that *CYP3A4* loss-of-function alleles can be classified as polymorphisms (i.e. with allele frequencies above 1%) and affect a substantial number of individuals, in specific populations/regions.

Haplotype analysis suggested that *CYP3A4*20* appeared in a haplotype present in only 4% of chromosomes of the Spanish population, and containing the most common Caucasian variants for the *CYP3A* SNPs genotyped (i.e. wild-type *CYP3A4*22, CYP3A4*1B* and *CYP3A7*2*, and variant *CYP3A5*3* allele; see Figure 2).[19] The highest frequency of *CYP3A4*20* in Spain and the infrequent detection outside the Spanish peninsula, together with a 700 kb haplotype common to all mutation carriers (Supplementary Figure 2), suggests a recent occurrence of the mutation. In agreement with this, dating of *CYP3A4*20* mutation suggested that it appeared about 1000 years ago. Altogether, these data are compatible with a single origin of the mutation in Spain, and then spreading to different geographical areas in recent times.

CYP3A4 has a prominent role in the biotransformation of a broad range of xenobiotics, including many clinical drugs,[14] and contributes to the metabolism of endogenous substrates such as vitamine D3, arachidonic acid, bile acids and steroid hormones.[2,20] It has been suggested that the *CYP3A4* gene allows little variation owing to this fact and only one individual being homozygous for defective *CYP3A4* alleles has been described.[11] With the exception of drug metabolism impairment, no other major phenotype could be detected in *Cyp3a* knockout mice.[21,22] Alternative enzymes may have compensated the effect on the metabolism of endogenous compounds, whereas the prominent decrease in xenobiotic biotransformation would only manifest after xenobiotic exposure. In contrast, in a human CYP3A4-transgenic mouse line, the females were found to be deficient in lactation, leading to a markedly lower pup survival, and the mammary glands of the Tg-CYP3A4 lactating mothers had underdeveloped alveoli with low milk content.[23] Because of the absence of a null phenotype in mice, the small number of individuals expected to be homozygous for *CYP3A4*20* (i.e. one in 4100 individuals in Castilla y León) might not have any clinical manifestation, although a very severe toxicity profile would be expected when exposed to drugs metabolized by this enzyme.[11] *CYP3A4*20* heterozygous carriers, with decreased CYP3A4 activity, may not show an effect when treated with single doses of wide therapeutic index drugs, but may show altered response upon treatment with narrow therapeutic index drugs. This is supported by Westlind-Johnsson *et al*.[9] who described a sixfold higher exposure to a drug metabolized by CYP3A4 and low systemic midazolam clearance in an individual heterozygous for the *CYP3A4*20* allele[9] and by a sevenfold higher risk of paclitaxel dose reductions because of peripheral neuropathy in the *CYP3A4*20* carriers as described by us.[15] We also found that *CYP3A4*20* is independent of *CYP3A4*22*, an intronic polymorphism robustly associated with a decreased elimination of CYP3A4 substrates and carried by about 5–7% of Caucasians.[13,24] Thus, a highly reduced CYP3A4 activity would be expected in individuals carrying both of these two alleles.

In conclusion, this is the first demonstration of a polymorphic nature of *CYP3A4* gene, with 1.2% of Spanish individuals carrying *CYP3A4*20* allele, likely due to a founder effect. Furthermore, the key role of CYP3A4 in drug metabolism and in preliminary clinical evidences support an increased risk of unexpected drug responses in *CYP3A4*20* carriers and suggest the importance of implementing *CYP3A4*20* genotyping in the clinic, at least in the Spanish population.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)

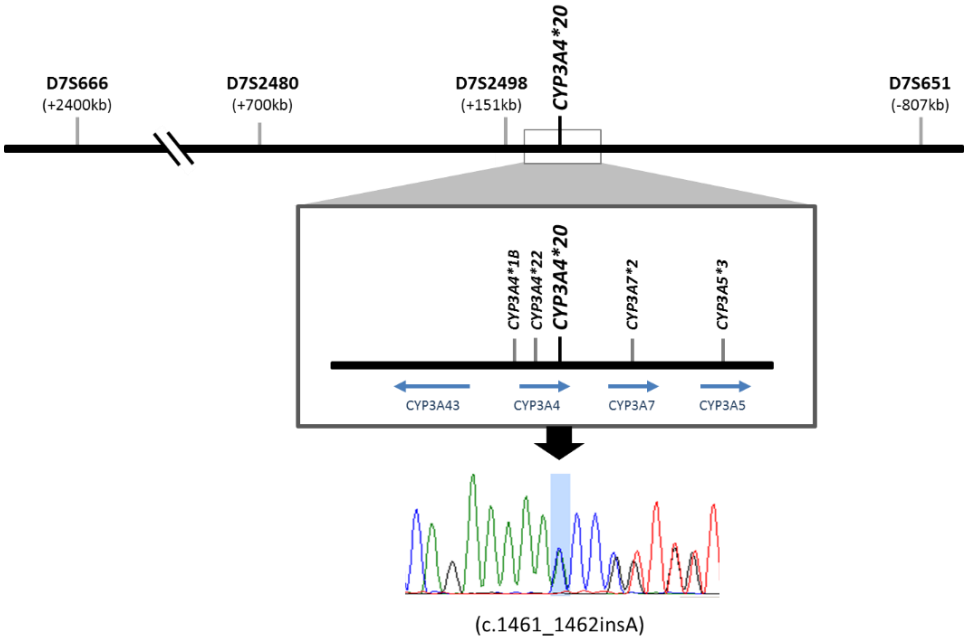
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Distribution of *CYP3A4*20* allele in Spain.

Region in Spain	*CYP3A4* wild type homozygous (Nr)	*CYP3A4*20* heterozygous (Nr)	*CYP3A4*20* carriers (%)
Castilla León	151	6	3.8
Comunidad Valenciana	129	4	3.0
Extremadura	142	3	2.1
Castilla la Mancha	62	1	1.6
Andalucia	113	1	0.9
Cataluña	126	1	0.8
Comunidad Madrid	508	4	0.8
Galicia	153	0	0.0
Other Spanish regions*	78	1	0.4
No birth place data	491	3	0.6
TOTAL	**1953**	**24**	**1.2**

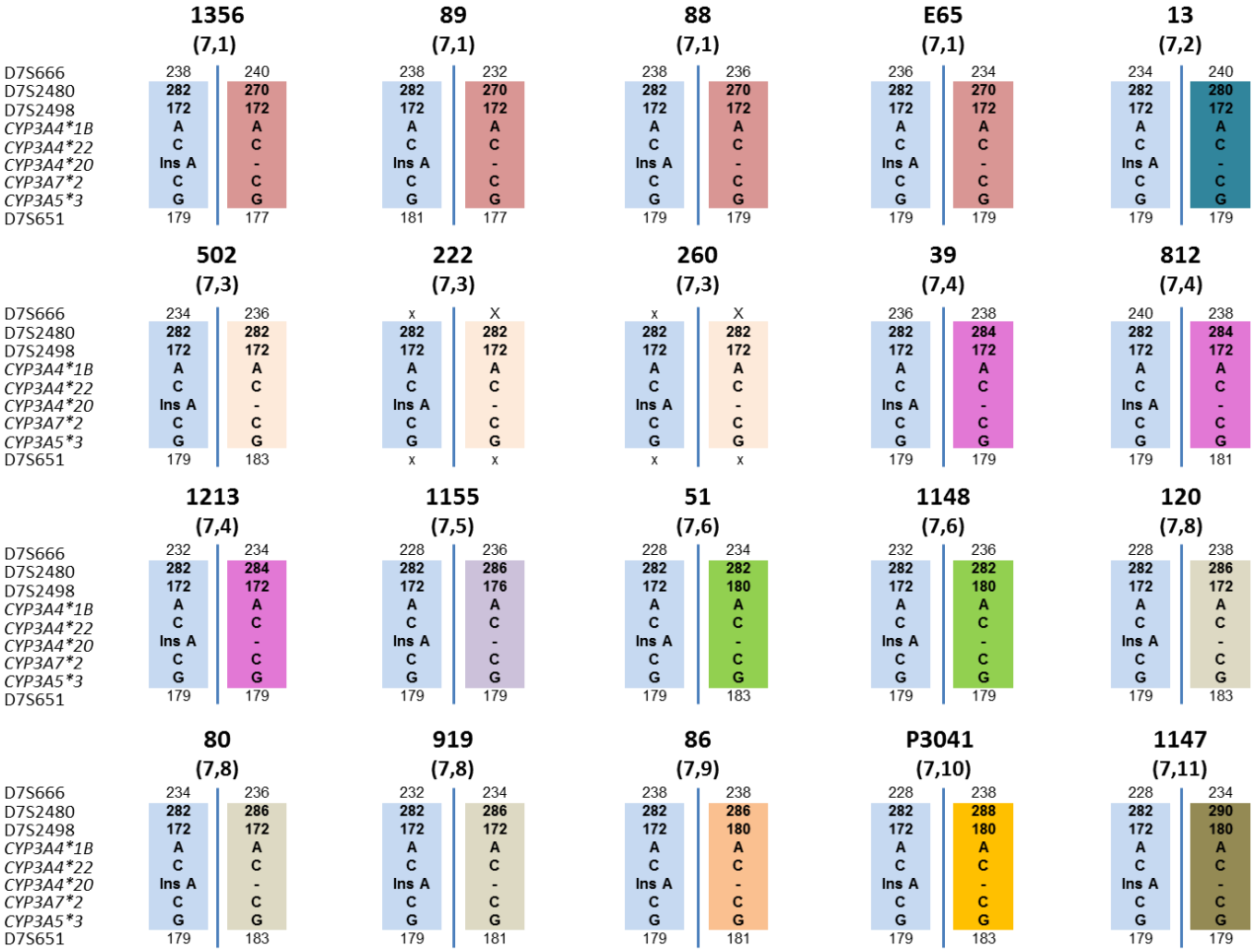
*Other Spanish regions: 57 from Murcia, 5 from Asturias, 4 from Navarra (1 of them *CYP3A4*20* carrier), 4 from País Vasco, 3 from Cantabria, 2 from Aragón, 2 from Ceuta y Melilla, 1 from Canarias and 1 from Islas Baleares.

Supplementary Figure 1



Representation of the microsatellites and SNPs used for haplotype analysis. Graph indicates the location of microsatellites (D7S651, D7S2498, D7S2480 and D7S666) and SNPs (*CYP3A4*22*, *CYP3A4*1B*, *CYP3A7*2* and *CYP3A5*3*) used for haplotype analysis. Microsatellites location is indicated with respect to *CYP3A4*20*. *CYP3A* locus is magnified indicating the orientation of the genes. Sanger sequencing of a *CYP3A4*20* (c.1461_1462insA) heterozygous variant is shown.

Supplementary Figure 2



Haplotypes of *CYP3A4*20* carriers. PHASE was used to determine the common haplotype in the 20 *CYP3A4*20* carriers used in the analysis. D7S651 and D7S666 were not amplified for samples 222 and 260. Recurring haplotypes are color shaded.

ARTICLE 5: Targeted exome sequencing identifies markers of chemotherapy-induced peripheral neuropathy

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Under review in Clinical Cancer Research

Abstract:

As previously mentioned, paclitaxel is a microtubule stabilizing agent widely used to treat solid tumors. However, paclitaxel-induced neuropathy can limit treatment success and importantly reduce the quality of life of the patients. Although polymorphisms in paclitaxel metabolizing enzymes, in paclitaxel targets and in *EPHA* genes have been associated with neuropathy, they only show moderate effects. In this study we proposed that low-frequency variants with strong effects, may also contribute to the variability in neuropathy susceptibility observed among patients. To test this hypothesis, we designed and performed a targeted exon sequencing analysis of 39 genes including *EPHA* family genes, genes involved in paclitaxel pharmacokinetics and genes causative of familial neuropathies (Charcot-Marie-Tooth). In total 228 patients with high or no/low paclitaxel-induced neuropathy were analyzed.

In the discovery series, *EPHA6* was the gene most significantly associated with paclitaxel-induced neuropathy (P=0.041). Interestingly, low frequency non-synonymous variants in *EPHA6* were present exclusively in patients with high neuropathy and all affected the ephrin receptor ligand binding domain. In addition, using cumulative paclitaxel dose analysis we observed a significantly higher neuropathy risk for carriers of *EPHA5/6/8* low-frequency non-synonymous variants. This association was further confirmed in an independent cohort of 202 patients treated with paclitaxel.

In conclusion, this is the first study identifying low-frequency variants in *EPHA6*, *EPHA5* and *EPHA8* as markers of paclitaxel-induced neuropathy susceptibility. This further support that these genes are relevant neuropathy markers for other neurotoxic agents.

Personal contribution: I participated in the selection of the patients, I processed the DNAs to create the next generation sequencing libraries and performed the targeted NGS using a MiSeq Illumina sequencer. I validated selected variants through Sanger sequencing. Moreover, I collaborated in the variant analyses (SKAT and Chi square tests). I generated the graphs to represent *EPHA* variants using Illustrator for Biological Sequences. Finally, I contributed to the discussion of the results and I was the principal author drafting the paper.

Targeted sequencing reveals low-frequency variants in *EPHA* genes as markers of paclitaxel-induced peripheral neuropathy

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Running head: *EPHA* as markers of paclitaxel induced neuropathy

Keywords: *EPHA*, neuropathy, paclitaxel, targeted next generation sequencing

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Conflicts of interest: No potential conflicts of interest were disclosed by the authors.

Translational Relevance

Paclitaxel treatment frequently causes peripheral neuropathy, an adverse event that can limit treatment course and lead to permanent symptoms drastically decreasing quality of life. Our group has contributed to the identification and validation of common polymorphisms in *EPHA* genes associated with paclitaxel neuropathy, but a large part of the inter-individual variation in neuropathy remains unexplained. We hypothesized that low-frequency variants with strong effects may contribute to the neuropathy variability in patients. By performing targeted exon sequencing of candidate genes we found that patients carrying low-frequency non-synonymous coding variants in *EPHA5/6/8* contribute to paclitaxel-induced neuropathy susceptibility. Furthermore, these genes might also be relevant neuropathy markers for other neurotoxic drugs due to the involvement of Eph receptors in neuronal functions.

ABSTRACT

Purpose: Neuropathy is the dose limiting toxicity of paclitaxel and a major cause for decreased quality of life. Genetic factors have been shown to contribute to paclitaxel neuropathy susceptibility; however, the major causes for inter-individual differences remain unexplained. In this study we identified genetic markers associated with paclitaxel-induced neuropathy through massive sequencing of candidate genes.

Experimental Design: We sequenced the coding region of 4 *EPHA* genes, 5 genes involved in paclitaxel pharmacokinetics and 30 Charcot-Marie-Tooth genes, in 228 cancer patients with no/low neuropathy or high grade neuropathy during paclitaxel treatment. An independent validation series included 202 paclitaxel-treated patients. Variation-/ gene-based analyses were used to compare variant frequencies among neuropathy groups and Cox regression models were used to analyze neuropathy evolution along treatment.

Results: Gene-based analysis identified *EPHA6* as the gene most significantly associated with paclitaxel-induced neuropathy. Low frequency non-synonymous variants in *EPHA6* were present exclusively in patients with high neuropathy and all affected the ligand binding domain. Accumulated dose analysis in the discovery series showed a significantly higher neuropathy risk for *EPHA5/6/8* low-frequency non-synonymous variant carriers (HR=18.04, 95%CI=2.87-113.36, P=0.0020) and an independent cohort confirmed an increased neuropathy risk (HR=1.96, 95%CI=1.08-3.56; P=0.028). Combining the series gave an estimated 2.81- fold higher risk of neuropathy (95%CI=1.63-4.86; $P=2.1x10^{-4}$).

Conclusion: This first study sequencing *EPHA* genes revealed that low frequency variants in *EPHA6, EPHA5* and *EPHA8* contribute to the susceptibility to paclitaxel-induced neuropathy. Furthermore, EPHAs neuronal injury repair function suggests that these genes might constitute important neuropathy markers for many neurotoxic drugs.

INTRODUCTION

The anticancer agent paclitaxel is a microtubule inhibitor widely used in the treatment of many solid tumors (1). Peripheral neuropathy is its dose-limiting toxicity (2), and severe neuropathy cases with an important reduction in the quality of life of the patients are not rare (3, 4). The lack of effective treatments for the neuropathy creates an urgent need to identify markers that can help to personalize treatment and avoid severe neuropathy events. The patient genetic background has been proposed to play a relevant role in the susceptibility for suffering neuropathy (5). In this regard, paclitaxel pharmacokinetic (6, 7) and pharmacodynamic (8, 9) pathways have been included in studies of candidate genes and, more recently genome-wide association studies (GWAS) have been performed (10, 11).

Candidate gene studies, by us and other groups, have demonstrated that common variants in paclitaxel metabolizing enzymes and paclitaxel target (i.e. *CYP2C8*3* (12-14), *CYP3A4*22* (7), *TUBB2A* rs909964 and rs909965 (8, 9)) influence neuropathy risk, while genome wide genotyping has uncovered novel genes (10, 11). A GWAS by our group (11) suggested that the *EPHA* gene family, which plays a key role in the development of nervous system and in nerve injury repair (15-17), was a key player for paclitaxel neuropathy susceptibility. Meta-analysis of GWAS top hits showed that *EPHA5* rs7349683 reached genome-wide significance (11), and follow-up studies further supported that this variant (18), *EPHA6* rs301927 (9, 18) and *EPHA8* rs209709 (18) moderately increased paclitaxel-induced neuropathy risk. However, large part of the variation in paclitaxel-induced neuropathy remains unexplained.

Low-frequency variants with strong effects may contribute to the neuropathy variability observed in patients. To investigate this hypothesis sequencing technologies are required and, so far, only two exploratory studies following different strategies have been performed. In one we applied whole exome sequencing to few extreme neuropathy patients, and identified defective *CYP3A4* variants associated with the neuropathy (19). The second study sequenced genes causative of familial polyneuropathies (Charcot-Marie-Tooth, CMT), and suggested *ARHGEF10* and *PRX* as chemotherapy-induced neuropathy markers (20). These initial studies are promising, however, the statistical power for a whole exome sequencing study is low and in the CMT analysis key genes were excluded.

Here, we performed targeted exome sequencing of genes with common variants associated with paclitaxel-induced neuropathy (*EPHA4, EPHA5*, *EPHA6* and *EPHA8*) plus genes involved in paclitaxel pharmacokinetics and in CMT. In total we sequenced 39 genes in 228 selected patients with high or no/low paclitaxel-induced neuropathy. The strongest association corresponded to *EPHA6*, and the relevance of low frequency *EPHA5/6/8* non-synonymous coding variants was validated in an independent cohort of 202 paclitaxel-treated patients. These results reveal *EPHA* genes as key players in chemotherapy-induced neuropathy and stress the importance of gene sequencing for identifying genetic risk factors of neuropathy.

PATIENTS AND METHODS

Patients

The discovery series was derived from a set of 449 breast or ovarian cancer patients treated with paclitaxel (97% in first line), with DNA available, no previous neurotoxic drug treatments and with clinical data and neuropathy assessment; some have already been reported (18, 19, 21). In these patients the neuropathy was homogenously graded (19), and 228 were selected for whole or targeted exon deep-sequencing, based on extreme-neuropathy phenotype. Among them, 131 were high-neuropathy patients that fulfilled the following criteria: grade 3 or 2 neuropathy (NCI-CTC v4) during paclitaxel treatment, no neuropathy risk factors (diabetes, alcoholism, AIDS or previous neuropathies), and treatment modifications due to neuropathy (dose reduction or treatment suspension) or neuropathy that lasted >6 months after paclitaxel treatment finished. The remaining 97 patients were no/low-neuropathy patients with no neuropathy signs or grade 1 neuropathy after receiving paclitaxel (Table 1).

The validation of results was performed in an independent series of 202 paclitaxel-treated patients with neuropathy data recorded cycle by cycle. Most patients had breast or ovarian tumors, 109 were Spanish (54%) and 93 Swedish (46%). 129 samples corresponded to a previous GWAS study (11), 37 to Spanish patients already described (18) and 36 samples were new cases collected in Spain. From all patients cumulative paclitaxel dose up to grade 2 (NCI_CTC v2/4) neuropathy was available (Table 1).

All individuals participating in the study were over 18 years of age, had been diagnosed of cancer with histological confirmation, a life expectancy of ≥12 weeks and ECOG performance status ≤2, adequate bone marrow and renal and hepatic function. The recruitment of patients and collection of samples was approved by local internal ethical review committees and all patients gave written informed consent to participate in the study.

Next generation sequencing (NGS)

From the 228 patients used in the discovery series, 196 samples were processed using the TruSeq Custom Amplicon Kit (Illumina) covering the coding plus 25 bp intronic flanking region of 39 genes that included: *EPHA4, EPHA5, EPHA6* and *EPHA8* (10, 11) plus additional genes involved in paclitaxel metabolism and transport (*ABCB1, CYP2C8*, *CYP3A4*, *SLCO1B1*, *SLCO1B3*) and a selection of 30 genes associated with CMT hereditary peripheral neuropathies (Fig. 1). Very conserved CMT genes with no/very few variants reported were not selected for sequencing (e.g. *ATL1, EGR2, GDAP1, GJB1, LMNA, PRPS1, RAB7A, YARS*). In brief, 150 ng of DNA extracted from peripheral blood (FlexiGene DNA Kit, Qiagen) was used to construct libraries and sequenced in a MiSeq sequencer (Illumina, Spain) with a paired-end mode using MiSeq Reagent Kit V3 (Illumina, Spain) and 600 cycles. In addition, whole exome sequencing was performed on the remaining 32 patients (16 with high neuropathy (8 have been reported (19)) and 16 patients with no neuropathy), as previously described (19).

For the validation of the results, a TruSeq Custom Amplicon Kit (Illumina) including the coding and intronic flanking region of *EPHA5*, *EPHA6* and *EPHA8* was used.

Variant identification

Targeted NGS data was demultiplexed with MiSeq Reporter (Illumina). Alignment was performed using Smith-Waterman algorithm (22) using GRCh37/hg19 assembly as reference and Genome Analysis Toolkit v2 (GATK, (23)) was used for raw variant calling. For the 32 samples with whole exome sequencing data, alignment and variant calling were performed by RUbioSeq software v3.7 (24). In this software the alignment was performed using Burrows-Wheeler alignment (25), unmapped reads are realigned using BFAST (26) and for variant calling, GATK v2 was used (23). Variants were annotated with Snp Eff (http://snpeff.sourceforge.net/) and Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep/index.html), and only non-synonymous coding variants and those altering canonical splice sites, with P>0.001 for Hardy Weinberg Equilibrium were considered in subsequent steps. Supplementary Table 1 indicates gene and transcript references.

Variants included in the analysis were: i) those previously described in public databases (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/; Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org), and ii) variants not previously described with: high variant call quality (Q>30), read depth >10X and alternative variant frequency higher than 0.3 in at least one individual. Sequencing artefacts, defined as nucleotide changes detected in >20 samples in the sequencing panel but never described in ExAC, were omitted from the analysis. We defined loss of function (LOF) variants as those introducing stop codons (nonsense), variants disrupting canonical splice sites and indels disrupting the reading frame. Template and configuration files for alignment and scripts are available at https://github.com/htejero/PaclitaxelNeuropathy.

Validation of variants was performed by Sanger sequencing with an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems) on 3% of the LOF and missense variants included in the analysis.

Data analysis

Variants were classified as “common variants” if they had a minor allele frequency (MAF) ≥0.5% in the more than 30.000 sequenced non-Finnish Europeans from ExAC. Variants were classified as “low frequency variants” if they had a MAF <0.5% in the non-Finnish Europeans from ExAC and MAF <1% in 578 Spanish exomes from the CIBERER Spanish Variant Server (http://csvs.babelomics.org/). The purpose of including the Spanish data was to detect population specific variants, because of the small sample size (n<600) the MAF threshold in this population was less stringent. For common variants, the frequency of each variant in the high versus no/low neuropathy group was compared with a Chi^2 or Fisher test.

For low frequency variants, the association with paclitaxel-induced neuropathy was assessed with the gene-based Burden test (27) using the SKAT package and R statistical software (http://www.R-project.org/). Scripts are available at https://github.com/htejero/PaclitaxelNeuropathy. Based on statistical power calculations, only genes with ≥ four rare variants were included in the analysis.

For samples with cycle by cycle neuropathy data, association between *EPHA* variants and paclitaxel neuropathy risk was tested using Kaplan-Meier analysis, modelling the cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity. Patients with no or low neuropathy (grade 0/1) were censored at total cumulative dose. We also evaluated the association using univariate and multivariable Cox regression analysis (14), the latter including country of origin as covariate. SPSS software package v.19 was used for these analyses. P values less than 0.05 were considered statistically significant.

RESULTS

Study population and NGS

NGS was performed on selected cases: 131 patients with high neuropathy (grades 2/3 that lasted a mean of 55 months) despite low accumulated paclitaxel dose (median= 1295 mg) and 97 patients with no/low neuropathy (grades 0/1) despite high accumulated paclitaxel dose (median= 1485 mg) (Table 1). In addition, 33% of patients in the high neuropathy group had paclitaxel dose reductions or treatment suspensions caused by the neuropathy.

Sequencing of 39 candidate genes in the 228 patients identified 277 coding non-synonymous or canonical splice site variants (266 missense, 3 in-frame deletions, 8 LOF; Suppl. Table 1). From these, 86 were common variants present in all genes except for *CYP3A4*, *EPHA4*, *HSPB1*, *HSPB8*, *NDRG1* and *SPTLC2* (which only had low frequency variants) and *NEFL* (with no variants detected). When the presence of these common variants was compared among the neuropathy groups, association with paclitaxel neuropathy was found for only 2 SNPs located in *CYP2C8* and *PRX* ($P<0.05$; Suppl. Table 2).

The remaining 191 variants were low frequency variants and were present in all genes except for *NGF* (with only common variants) and *NEFL* (with no variants). Among these, 3 altered canonical splice sites, 2 were nonsense variants and 3 were indels causing frameshifts leading to premature stop codons (Table 2).

Gene-based analysis of paclitaxel-induced neuropathy

Analysis of the low frequency variants identified *EPHA6* as the gene most significantly associated with paclitaxel-induced neuropathy (Table 3). The 5 carriers of these variants were all high neuropathy patients with an amino acid change in the ephrin receptor ligand binding domain of the protein. Remarkably, no *EPHA6* variant carriers were present in the no/low-neuropathy group, suggesting a strong effect on neuropathy.

One additional gene had this characteristic (*SEPT9*), but results did not reach statistical significance level. The other two *EPHA* genes analyzed, *EPHA5* and *EPHA8*, have a similar biological function as *EPHA6* (15-17) and also belonged to the high-neuropathy risk group of genes (Table 3). In *EPHA5*, 5 carriers had high neuropathy versus 1 with low neuropathy; and in *EPHA8*, 9 carriers were in the high neuropathy and 6 in the no/low neuropathy group (Fig. 2; Suppl. Table 1). The highly conserved *EPHA4*, with only 2 carriers, one in each group, could not be analyzed.

Some of the discovery series patients had cycle by cycle neuropathy data available and among these, 3 were carriers of low-frequency variants in *EPHA5/6/8* genes (one variant in each gene). Accumulated paclitaxel dose analysis revealed that these patients had a significantly higher risk to suffer from neuropathy than patients without *EPHA* low frequency variants (HR=18.04, 95%CI=2.87-113.36, P=0.0020; Fig. 3A).

Low frequency variants in *EPHA6, EPHA5* and *EPHA8* confirmed as neuropathy risk factor

Sequencing *EPHA5/6/8* in an independent cohort of 202 patients treated with paclitaxel and detailed cycle by cycle neuropathy data (Table 1), revealed 15 carriers of low frequency missense variants in these genes (one corresponded to *EPHA6,* one to *EPHA5* and 13 to *EPHA8*). These variants were combined and an accumulated paclitaxel dose analysis revealed that low frequency *EPHA5/6/8* variants conferred increased risk of neuropathy (HR=1.96, 95%CI=1.08-3.56, P=0.028; Fig. 3B). Combining discovery and validation series, resulted in a HR of 2.81 (95%CI=1.63-4.86) with a P value of 2.11×10^{-4} (Fig. 3C).

DISCUSSION

Paclitaxel induced-neuropathy is a clinically relevant toxicity affecting large number of cancer patients. Genetic variation has been shown to influence susceptibility to paclitaxel-induced neuropathy, however, a large part of the variation remains unexplained. Low-frequency variants with strong effects may explain part of the variability. To investigate this hypothesis, we performed massive sequencing of candidate genes in patients selected based on extreme-neuropathy phenotype. Gene-based analysis identified, for the first time, low frequency genetic variants in *EPHA5/6/8* as risk factors of chemotherapy induced neuropathy. These results may provide a basis for personalizing paclitaxel treatment and decreasing the incidence of severe chemotherapy-induced neuropathies.

GWAS studies have identified common variants in *EPHA* genes with moderate effects on paclitaxel-induced neuropathy (E*PHA5*-rs7349683, *EPHA6*-rs301927, *EPHA8*-rs209709 and *EPHA4*-rs17348202) (10, 11) and subsequent studies further supported the association of *EPHA5, EPHA6* and *EPHA8* polymorphisms (9, 18). Non-synonymous coding variants, potentially affecting protein function, are expected to have stronger effects on neuropathy than common regulatory variants (28). Following this idea, we performed a NGS study in *EPHA* genes, together with paclitaxel pharmacokinetics and hereditary peripheral neuropathy related genes.

Gene-based analysis of our data revealed that low frequency missense variants in *EPHA6* increased paclitaxel-induced neuropathy risk. All these variants were located in the ephrin receptor ligand binding domain, suggesting an alteration of the protein function and further supporting the association. *EPHA5* and *EPHA8* followed a similar trend (Fig. 2). In total, 15% (19 of 131) of patients in the high neuropathy group carried low frequency non-synonymous coding variants in *EPHA5/6/8* genes. In the 202 patients of the validation series, 13 *EPHA8* variant carriers were identified but only one *EPHA6* and one *EPHA5* carriers were detected, suggesting that *EPHA6* and *EPHA5* variants (present in 5 out of the 131 patients with high-neuropathy of the discovery) are less frequent in an unselected patient population, including many moderate-neuropathy patients (not represented in the discovery set). Thus, *EPHA6* and *EPHA5* variant carriers were scarce in the validation series, and the calculated EPHA-effect mainly derived from *EPHA8*. Despite this, the accumulated dose analysis is a sensitive approach (18, 21) and was able to detect a statistically significant association. Altogether, these data suggest a relevant role for *EPHA5/6/8* genes in paclitaxel-induced neuropathy and indicates a high impact of low frequency variants missed in GWAS.

Eph receptors are tyrosine kinases involved in neural development (15) and nerve regeneration after damage (17, 29) among other functions: EphA4 controls axon sprouting/ nerve regeneration after spinal cord injury (30-32); EphA5 plays an important role in the initiation of the early phases of synaptogenesis (33) and it has been found upregulated in mice with injured sciatic nerve (34); EphA6 is involved in neural circuits underlying aspects of learning and memory (35); and EphA8 induces neurite outgrowth through induction of sustained MAPK activity (36) while lack of this gene produces aberrant axonal projections (37). Knocking out EphA4, EphA5, Eph6 and EphA8 genes in mice, results in viable and fertile animals with different neurological phenotypes. EphA4 knockout mice have gross motor dysfunction (38-40) and altered axonal regeneration and functional recovery following spinal cord injury (41). Knocking-out the tyrosine kinase domain of EphA5 results in axon aberrations in topographic mapping and altered behavioral patterns (42, 43). EphA8 knockout mice have abnormal axonal projections in the spinal cord (37) and EphA6 knockout mice experienced behavioral deficits in learning and memory tests (35). Thus, these are crucial genes for neural development and nerve regeneration with a plausible link for the association found with paclitaxel-induced neuropathy.

In ExAC database 0.1% of the European non-Finish population are carriers of LOF variants in either *EPHA5*, *EPHA6* or *EPHA8*, and on >100,000 Islandic individuals, two complete human knockouts for *EPHA5* and one for *EPHA6* were identified (44). So far, no phenotype has been assigned to these individuals who are apparently healthy subjects. However, based on the literature and on our results, a high susceptibility to drug-induced neuropathy would be expected.

Concerning other genes potentially associated with the neuropathy, in line with Beutler *et al* (20) we postulated that variants moderately affecting the function of CMT genes, while not being pathogenic, may increase the susceptibility to drug-induced neuropathy.

We did not find low frequency variants in *PRX* and common variants in *ARHGEF10* associated with paclitaxel-induced neuropathy, although the 2nd and 3rd top protective genes were these two, similarly to Beutler *et al*. For the *ARHGEF10* common variant rs9657362 we also found a trend towards protection (20, 45). We also observed a trend towards increased neuropathy risk for other CMT genes (*SEPT9* and *SH3TC2*). Variability in results among studies may be related to differences in neuropathy definitions/ assessments, in tumor types and patient treatments, or in the distribution of low-frequency variants, which have shown to be population-specific. Thus, results need to be further explored and validated in large independent series.

With regards to the LOF variants detected in this study, three occurred in CMT genes (*ARGHEF10, IKBAP* and *DHTKD1).* The patients with variants in *ARGHEF10* and *IKBAP* belonged to the no/low neuropathy group, in agreement with the fact that activating rather than LOF mutations in *ARGHEF10* cause CMT (46) and that no phenotype is observed for *IKBAP* heterozygous individuals (47). The variant in *DHTKD1* was present in two patients with different neuropathy, but recent data question the role of this gene in CMT disease (48, 49). Among the remaining LOF variants, two affected *EPHA* genes *(EPHA5* and *EPHA8)* and corresponded to high-neuropathy patients. One LOF occurred in the paclitaxel uptake transporter *SLCO1B1*, in a high neuropathy patient. Two occurred in *CYP3A4*, a gene in which we have demonstrated that defective variants increased neuropathy risk (19). Two patients were carriers of the *CYP3A4*20* frameshift allele and belonged to the high-neuropathy group, but one patient with a splicing defect affecting the last exon belonged to the no/low neuropathy group. The effect of this latter variant on the splicing of the gene and how it affects function remains to be studied.

Limitations of this study include gene selection, since relevant genes not yet connected with neuropathy susceptibility may have been excluded. There are also differences in the selection of patients in the discovery and validation series and, as mentioned before, although the number of patients in the study is substantial and the neuropathy assessment was homogenously performed to reduce subjectivity (11, 19), detection of low/ moderate effects on neuropathy may require even larger samples sets.

In conclusion, this study proves a relevant role of *EPHA5*, *EPHA6* and *EPHA8* genes in paclitaxel-induced neuropathy susceptibility and suggests that sequencing studies, rather genotyping, would be adequate approaches to study genetic markers of neuropathy. Moreover, taking into account the role of these proteins in neural development and injury repair, *EPHA* variants may also confer increased neuropathy risk to many additional neurotoxic drugs. The final goal is to identify genetic risk factors that can help to personalize neurotoxic drug treatments and avoid severe chemotherapy-induced neuropathies that can seriously affect patients' quality of life.

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TABLES

Table 1. Characteristics of the patients in the discovery (n=228) and validation (n=202) series.

Characteristics	Discovery series		Validation series
	High neuropathy	No/ low neuropathy	Cycle by cycle neuropathy data
Number of patients	131	97	202
Age (years)			
Median (min-max)	54 (35-82)	48 (32-73)	60 (34-82)
Gender			
Female	131 (100%)	97 (100%)	187 (93%)
Male	0 (0%)	0 (0%)	15 (7%)
Tumor type			
Breast	121 (92%)	82 (85%)	47 (23%)
Ovary	10 (8%)	15 (15%)	120 (60%)
Others	0 (0%)	0 (0%)	35 (17%)
Type of paclitaxel treatment			
First line	129 (99%)	95 (98%)	192 (95%)
Second line[a]	2 (1%)	2 (2%)	10 (5%)
Paclitaxel treatment[b]			
FEC+T	81 (62%)	23 (24%)	0 (0%)
AC+T	18 (14%)	18 (19%)	35 (17%)
T+FEC	14 (11%)	29 (30%)	0 (0%)
C+T	10 (7%)	15 (15%)	156 (77%)
Others	8 (6%)	12 (12%)	11 (6%)
Number of paclitaxel cycles			
Median (min-max)	8 (3-13)	10 (6-27)	7 (2-44)
Paclitaxel accumulated total dose (mg)			
Median (min-max)	1295 (450-1600)	1485 (900-4059)	1225 (114-3150)
Maximum sensory neuropathy grade[c]			
Grade 0	0 (0%)	56 (58%)	32 (16%)
Grade 1	0 (0%)	41 (42%)	42 (21%)
Grade 2	30 (23%)	0 (0%)	78 (38%)
Grade 3	101 (77%)	0 (0%)	50 (25%)
Dose modifications due to neuropathy[d]			
Paclitaxel dose reduction	14 (11%)	0 (0%)	21 (10%)
Paclitaxel treatment suspension	29 (22%)	0 (0%)	23 (11%)

[a] Patients with second line paclitaxel treatment and no previous neurotoxic drugs in first line therapy.

[b] Some patients receiving chemotherapeutic drugs in combination with targeted therapy (bevacizumab, trastuzumab, denosumab or pertuzumab) are included in the table according to the chemotherapy agents received. FEC+T: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2, every 7 days. AC+T: doxorubicin 60mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 80mg/m^2, every 7 days. T+FEC: paclitaxel 80 mg/m^2, every 7 days, followed by 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m2, every 21 days. C+T: carboplatin AUC5-6 and paclitaxel 175mg/m^2, every 21 days.

[c] NCI-CTC v2/4.

[d] When in the same patient paclitaxel dose was first reduced and later on paclitaxel treatment was suspended, the patient is included in the table as "treatment suspension".

Table 2. Loss of function variants in the discovery series.

Gene	Type of gene	Variant[a]	Protein change	Nr individuals, Status	Discovery series group	Variant ID [b]	ExAC browser MAF [b]
ARHGEF10	CMT	c.1521_1522delAT[c]	p.Ala509His fs*515	1, Heterozygous	No/low NP	rs765378810	0.000066
IKBKAP		c.150+1G>A[c]	Splicing defect	1, Heterozygous	No/low NP	-	-
DHTKD1		c.1160-1G>C[c]	Splicing defect	2, Heterozygous	Both	rs760767010	0.000017
EPHA5	GWAS	c.2722dupT	p.Tyr908Leu fs*921	1, Heterozygous	High NP	-	-
EPHA8		c.1822C>T	p.Gln608*	1, Heterozygous	High NP	-	-
CYP3A4	PK	c.1461_1462insA	p.Pro488Thr fs*494	2, Heterozygous	High NP	rs67666821	0.00028
CYP3A4		c.1417-1G>C	Splicing defect	1, Heterozygous	No/low NP	rs141749477	0.0000083
SLCO1B1		c.1738C>T	p.Arg580*	1, Heterozygous	High NP	rs71581941	0.0016

[a] Genomic position and reference transcript are indicated in Supplemental Table 1.
[b] Variants not present in ExAC browser are indicated by "-".
[c] Variants not present in CMT databases (Inherited Peripheral Neuropathies Mutation Database http://www.molgen.vib-ua.be/CMTMutations/Mutations/MutByGene.cfm and OMIM http://www.omim.org/).
CMT: Charcot-Marie-Tooth; GWAS: Genome Wide Association Study; PK: pharmacokinetics; NP: neuropathy; MAF: minor allele frequency.

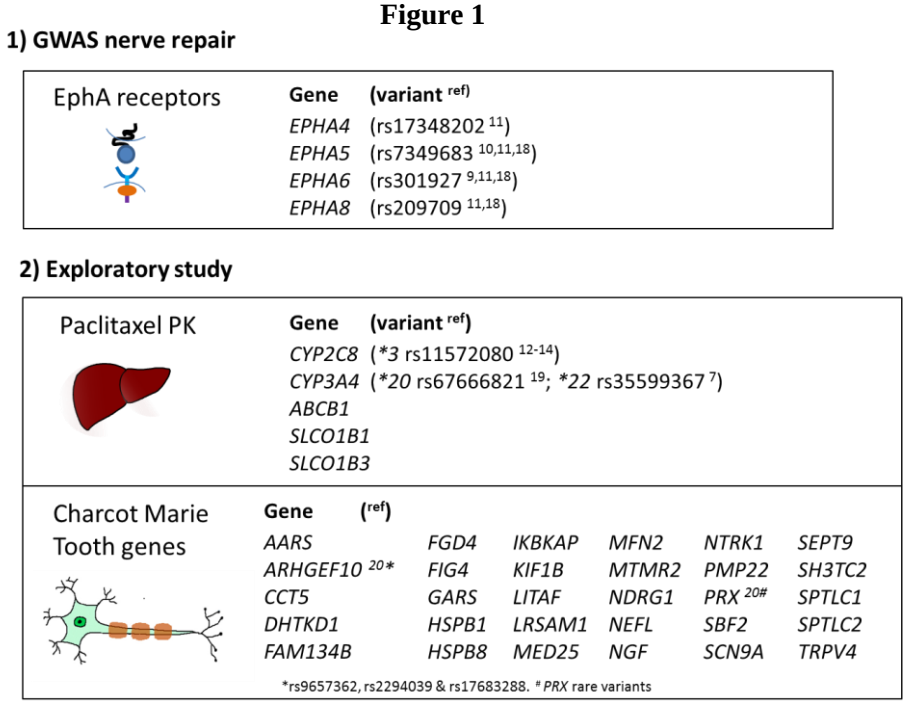
Table 3. Genes associated with paclitaxel-induced neuropathy using the gene-based burden test.

Gene	P-value	Number of variants carriers (variants)[a]	
		High neuropathy group, n=131	No/ low neuropathy group, n=97
Neuropathy risk			
EPHA6	0.041	5 (T72A,N127H,R162T,V196L)	0
SEPT9	0.072	4 (S96L,T235I,D348N,R355W)	0
SH3TC2	0.081	14 (T27A,V230A,T366A,S433L,Y510S,A590T,R658H,H696R,T755I,S831N,T1098P,D1229V)	4 (V230A,P251S,T1098P,D1229V)
EPHA5	0.219	5 (A49S,R494C,A611T,E678V,Y908fs)	1 (R238Q)
DHTKD1	0.271	9 (E42G,N107I,S114P,Q138K,A210S,c.1160-1G>C,T461K,I762del)	3 (I386V,c.1160-1G>C,G729R)
MFN2	0.323	6 (N63H,G298R,T423A,R468H,R663C)	2 (R468H,R707W)
LRSAM1	0.596	6 (I228M,F253V,Q409E,L500F,Q573K,L639P)	3 (S183L,R594C,Q697R)
SLCO1B3	0.737	5 (R23C,S64T,N145S,V235M)	3 (F36L,N145S,T414I)
ABCB1	0.752	5 (N183S,I261V,K624R,V835L)	3 (I261V,S1141T,R1225P)
EPHA8	0.785	9 (P321L,V365M,V444M,E462G,E464G,L559F,Q608*,A791V,D940H)	6 (G160S,I360V,V365M,E462G,Q525R,R679Q)
SBF2	0.787	7 (E304K,P339L,S730A,G775S,R890G,E1401K,K1672del)	3 (D289E,T1253S,A1849V)
SLCO1B1	0.800	4 (T10I,L193I,R580*,I656V)	3 (L193I,G210V)
Neuropathy protection			
TRPV4	0.082	1 (A293D)	4 (R160Q,R391W,T504A,S824L)
PRX	0.138	3 (M670V,P756L,D1013N)	6 (M670V,S751P K1062N,G1257R,E1360del,E1394D)
ARHGEF10	0.154	4 (S688N,H733Y,T811N,H1197Y)	7 (A509Hfs,S688N,H733Y,H834R,P956L,A960P)
NTRK1	0.261	2 (L79Q,G192A)	4 (L247P,Q570R,G714S,A779G)
SCN9A	0.456	4 (K40E,K655R,V1428I,L1916F)	5 (P74H,T152N,K655R,D1219E,L1267V)
IKBKAP	0.571	3 (M182K,R629H,G1013S)	4 (c.150+1G>A,M182K,S339R,R629H)
GARS	0.654	4 (C41R,R101H,S470F,T587M)	5 (T268I)
FAM134B	0.701	3 (P6L,V156F,S382T)	4 (M185V,V203M,Q379E,S382T)
Equal risk and protection			
AARS	0.650	5 (P234S,G275D,I579M)	5 (K81E,P234S,G275D,I579M)
FIG4	0.693	3 (I41T,K278N)	3 (I51V,A397P,E734K)
FGD4	0.712	3 (T79I,S392T,V717M)	3 (R275Q,V461A,D521G)
CYP3A4	0.795	4 (T185S,P389S,P488fs)	4 (R130Q,R162Q,T363M,c.1417-1G>C)

[a] Genomic position and reference transcript are indicated in Supplemental Table 1.

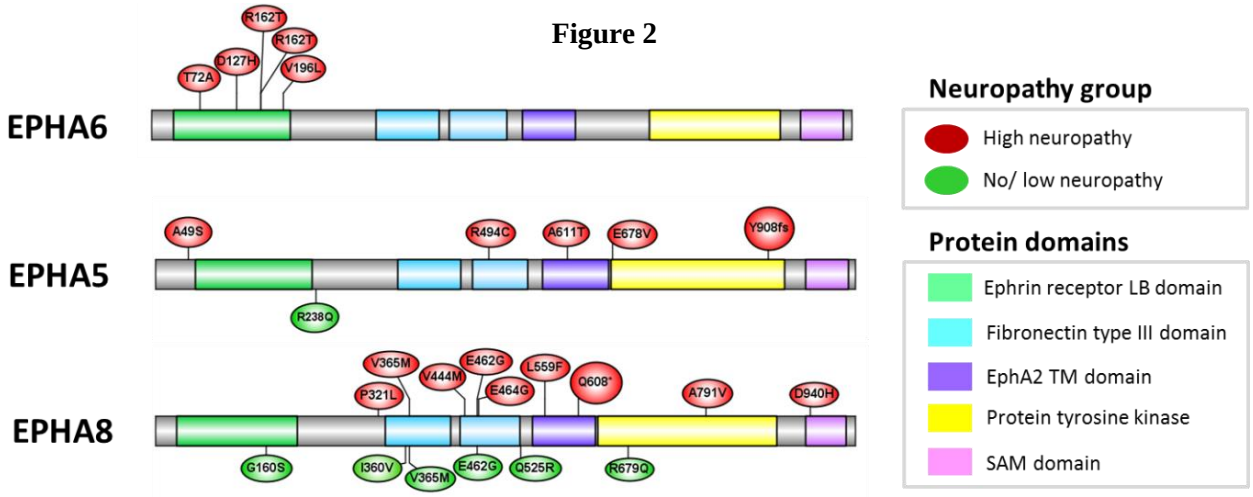
FIGURES

Figure 1



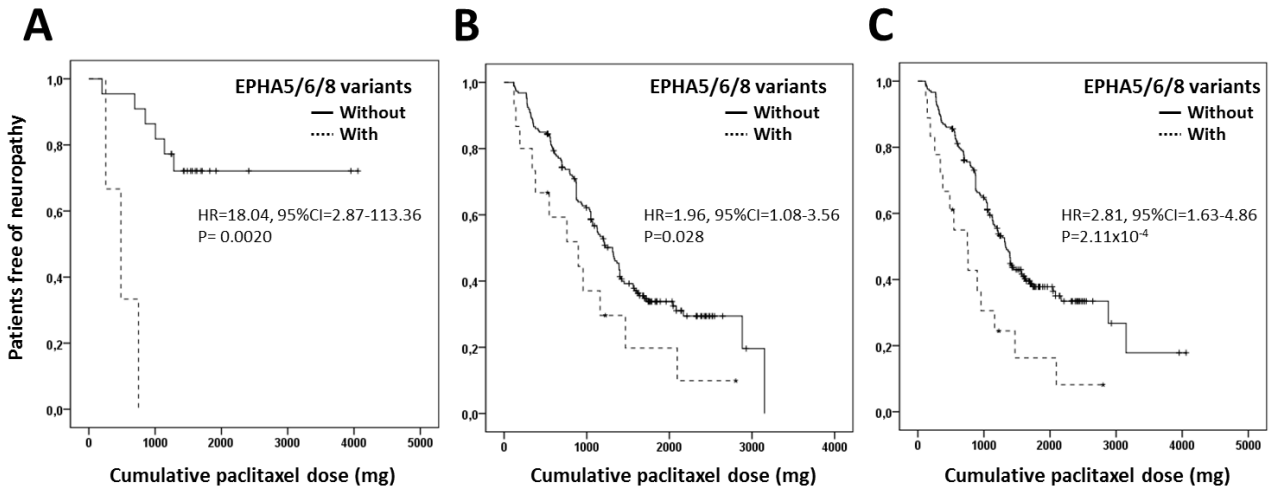
Genes selected for targeted NGS. The NGS panel included 39 genes classified into two categories: 1) four *EPHA* genes involved in neural processes and found to be associated with taxane-induced neuropathy through GWAS; 2) 35 additional genes selected for an exploratory study, involved in paclitaxel pharmacokinetic (PK) or causative of Charcot-Marie-Tooth. Variants previously described to be associated with paclitaxel-induced neuropathy are included in the graph and the corresponding references provided.

Figure 2



Non synonymous *EPHA* coding variants in the discovery series. The low frequency variants in *EPHA6*, *EPHA5* and *EPHA8* are represented along the protein sequences. In red variants in the high neuropathy group; in green variants in the no/low neuropathy group of patients. Protein domains are depicted according to Pfam database. Illustrator for Biological Sequences was used to create the graphs (http://ibs.biocuckoo.org/).

Figure 3



Kaplan-Meier analysis of paclitaxel-induced neuropathy. Patients were grouped according to the absence (Without) or presence (With) of low-frequency variants in *EPHA5*, *EPHA6*, and *EPHA8*, and the cumulative dose of paclitaxel up to the development of grade 2 peripheral sensory neuropathy was compared. A) Discovery series (n=25). B) Validation series (n=202). C) Analysis combining patients from discovery and validation series (n=227). P values correspond to multivariable Cox regression analyses including country of origin as covariate.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Non synonymous coding and splicing site variants in the discovery series targeted sequencing.

Gene (Gene ID)	Chr:position	Variant ID	Ref	Alt	Transcript ID	Consequence	Protein change	MAF ExAC[a]	MAF Spanish[b]	High Neuropathy group (nr.)			No/Low Neuropathy group (nr.)			HWE (Yes /No) [a]
										wt wt	wt var	var var	wt wt	wt var	var var	
AARS (ENSG00000090861)	16:70286631	rs35744709	T	A	ENST00000261772	missense	Lys967Met	0.01467	0.014	124	7	0	93	4	0	Yes
	16:70286740	rs149377346	C	T		missense	Gly931Ser	0.01102	0.010	128	3	0	94	3	0	Yes
	16:70287883	rs147319762	T	C		missense	Lys820Arg	0.00182	0.014	129	2	0	94	3	0	Yes
	16:70294995	rs144323646	G	C		missense	Ile579Met	0.00009	0.004	130	1	0	96	1	0	Yes
	16:70303659	rs11537667	C	T		missense	Gly275Asp	0.00038	0.004	130	1	0	96	1	0	Yes
	16:70304215	rs141840552	G	A		missense	Pro234Ser	0.00171	0.007	128	3	0	95	2	0	Yes
	16:70310961	-	T	C		missense	Lys81Glu	-	-	131	0	0	96	1	0	Yes
ABCB1 (ENSG00000085563)	7:87133728	rs779103120	C	G	ENST00000265724	missense	Arg1225Pro	-	-	131	0	0	96	1	0	Yes
	7:87138659	rs2229107	A	T		missense	Ser1141Thr	0.00009	-	131	0	0	96	1	0	Yes
	7:87138760	rs55852620	T	G		missense	Gln1107Pro	0.00582	0.002	129	2	0	97	0	0	Yes
	7:87160792	-	C	G		missense	Val835Leu	-	-	130	1	0	97	0	0	Yes
	7:87175195	rs141018820	T	C		missense	Lys624Arg	0.00007	0.001	130	1	0	97	0	0	Yes
	7:87179809	rs2229109	C	T		missense	Ser400Asn	0.04316	0.023	124	7	0	87	10	0	Yes
	7:87190625	rs36008564	T	C		missense	Ile261Val	0.00120	0.002	129	2	0	96	1	0	Yes
	7:87195540	rs60419673	T	C		missense	Asn183Ser	0.00167	0.002	130	1	0	97	0	0	Yes
	7:87229440	rs9282564	T	C		missense	Asn21Asp	0.11199	0.053	112	19	0	80	16	1	Yes
ARHGEF10 (ENSG00000104728)	8:1833801	rs9657362	G	C	ENST00000349830	missense	Leu370Phe	0.13846	0.119	110	19	2	71	26	0	Yes
	8:1844578	rs765378810	CAT	C		**frameshift**	Ala509Hisfs	0.00000	-	131	0	0	96	1	0	Yes
	8:1857556	rs143290224	G	A		missense	Ser688Asn	0.00105	0.007	130	1	0	96	1	0	Yes
	8:1857591	rs2294039	G	A		missense	Val700Ile	0.04063	0.055	123	7	1	88	8	1	Yes
	8:1871183	rs147531758	C	T		missense	His733Tyr	0.00176	0.003	130	1	0	96	1	0	Yes
	8:1871984	-	C	A		missense	Thr811Asn	0.00000	-	130	1	0	97	0	0	Yes

	8:1873461	rs142973221	A	G		missense	His834Arg	0.00006	-	131	0	0	95	2	0	Yes
	8:1876707	rs61752020	G	A		missense	Val938Ile	0.00817	0.011	129	2	0	94	3	0	Yes
	8:1876762	rs201570359	C	T		missense	Pro956Leu	0.00000	-	131	0	0	96	1	0	Yes
	8:1876773	-	G	C		missense	Ala960Pro	0.00003	-	131	0	0	96	1	0	Yes
	8:1877480	rs17683288	T	G		missense	Ser984Ala	0.07288	0.057	111	20	0	87	10	0	Yes
	8:1904983	rs200779877	C	T		missense	His1197Tyr	0.00055	-	130	1	0	97	0	0	Yes
CCT5 (ENSG00000150753)	5:10256172	rs11557652	A	T	ENST00000280326	missense	Glu146Val	0.02393	0.012	128	3	0	95	2	0	Yes
	5:10256175	rs118203986	A	G		missense	His147Arg	0.00002	-	131	0	0	96	1	0	Yes
	5:10256192	rs563305570	G	A		missense	Asp153Asn	0.00002	-	130	1	0	97	0	0	Yes
	5:10261764	rs141675330	C	G		missense	Ile362Met	0.00511	0.004	130	1	0	96	1	0	Yes
CYP2C8 (ENSG00000138115)	10:96797034	rs143038562	G	A	ENST00000371270	missense	Arg442Cys	0.00008	-	130	1	0	97	0	0	Yes
	10:96798749	rs10509681	T	C		missense	Lys399Arg	0.11299	0.152	87	39	5	64	29	4	Yes
	10:96818106	rs11572103	T	A		missense	Ile269Phe	0.00397	0.003	129	2	0	97	0	0	Yes
	10:96818119	rs1058930	G	C		missense	Ile264Met	0.05429	0.059	116	15	0	76	19	2	Yes
	10:96824658	rs41286886	C	T		missense	Val181Ile	0.00762	0.001	130	1	0	96	1	0	Yes
	10:96827030	rs11572080	C	T		missense	Arg139Lys	0.11287	0.137	92	35	4	67	27	3	Yes
CYP3A4 (ENSG00000160868)	7:99355806	rs67666821	G	GT	ENST00000336411	**frameshift**	Pro488fs	0.00034	-	129	2	0	97	0	0	Yes
	7:99355852	rs141749477	C	G		**splice accept**	c.1417-1G>C	0.00000	-	131	0	0	96	1	0	Yes
	7:99359752	rs749459749	G	A		missense	Pro389Ser	0.00002	-	130	1	0	97	0	0	Yes
	7:99359829	rs67784355	G	A		missense	Thr363Met	0.00022	-	131	0	0	96	1	0	Yes
	7:99366093	rs12721627	G	C		missense	Thr185Ser	0.00000	-	130	0	1	97	0	0	Yes
	7:99367427	rs4986907	C	T		missense	Arg162Gln	0.00019	0.002	131	0	0	96	1	0	Yes
	7:99367788	rs72552799	C	T		missense	Arg130Gln	0.00164	0.001	131	0	0	96	1	0	Yes
DHTKD1 (ENSG00000181192)	10:12111090	rs1279138	T	C	ENST00000263035	missense	Phe20Leu	0.00020	0.363	18	0	113	10	0	87	No
	10:12111157	-	A	G		missense	Glu42Gly	-	-	130	1	0	97	0	0	Yes
	10:12123525	rs34644609	C	G		missense	Ala70Gly	0.00684	0.008	128	3	0	96	1	0	Yes
	10:12126548	-	A	T		missense	Asn107Ile	-	-	130	1	0	97	0	0	Yes
	10:12126568	-	T	C		missense	Ser114Pro	-	-	130	1	0	97	0	0	Yes
	10:12126640	-	C	A		missense	Gln138Lys	-	-	130	1	0	97	0	0	Yes
	10:12129639	rs146741810	G	T		missense	Ala210Ser	0.00403	-	129	2	0	97	0	0	Yes
	10:12131081	rs3740015	T	G		missense	Tyr272Asp	0.59906	0.427	23	53	55	14	56	27	Yes

	10:12133603	rs147571909	T	C		missense	Val360Ala	0.00558	0.003	130	1	0	96	1	0	Yes
	10:12133680	-	A	G		missense	Ile386Val	-	-	131	0	0	96	1	0	Yes
	10:12136071	rs760767010	G	C		**splice accept**	c.1160-1G>C	0.00002	-	130	1	0	96	1	0	Yes
	10:12139706	rs201559023	C	A		missense	Thr461Lys	0.00005	0.001	130	1	0	97	0	0	Yes
	10:12143105	rs2062988	C	G		missense	Ile607Met	0.81243	0.168	5	41	85	3	31	63	Yes
	10:12154929	rs117225135	G	A		missense	Gly729Arg	0.00233	0.004	131	0	0	96	1	0	Yes
	10:12155027	-	CATT	C		inframe del	Ile762del	-	-	130	1	0	97	0	0	Yes
EPHA4 (ENSG00000116106)	2:222294690	rs142860268	G	A	ENST00000281821	missense	Thr893Met	0.00007	0.003	131	0	0	96	1	0	Yes
	2:222365859	rs200225096	G	A		missense	Thr286Met	0.00010	-	130	1	0	97	0	0	Yes
EPHA5 (ENSG00000145242)	4:66201779	-	T	TA	ENST00000273854	**frameshift**	Tyr908fs	-	-	130	1	0	97	0	0	Yes
	4:66231667	-	T	A		missense	Glu678Val	-	-	130	1	0	97	0	0	Yes
	4:66231686	rs36050417	C	T		missense	Ala672Thr	0.02758	0.038	124	7	0	91	6	0	Yes
	4:66242741	rs777294375	C	T		missense	Ala611Thr	0.00002	-	130	1	0	97	0	0	Yes
	4:66286206	rs138678484	G	A		missense	Arg494Cys	0.00034	0.004	130	1	0	97	0	0	Yes
	4:66467556	rs147719164	C	T		missense	Arg238Gln	0.00016	-	131	0	0	96	1	0	Yes
	4:66509085	rs33932471	T	G		missense	Asn81Thr	0.06647	0.065	115	16	0	88	9	0	Yes
	4:66535316	rs138631715	C	A		missense	Ala49Ser	0.00033	0.001	130	1	0	97	0	0	Yes
EPHA6 (ENSG00000080224)	3:96533681	rs373432052	A	G	ENST00000389672	missense	Thr72Ala	0.00015	-	130	1	0	97	0	0	Yes
	3:96533846	rs200313366	A	C		missense	Asn127His	0.00037	-	130	1	0	97	0	0	Yes
	3:96706208	rs192891419	G	C		missense	Arg162Thr	0.00319	0.003	129	2	0	97	0	0	Yes
	3:96706309	-	G	T		missense	Val196Leu	-	-	130	1	0	97	0	0	Yes
	3:97311483	rs4857276	C	T		missense	Ala805Val	0.00742	0.011	129	2	0	97	0	0	Yes
EPHA8 (ENSG00000070886)	1:22895820	rs45498698	G	A	ENST00000166244	missense	Gly45Ser	0.00821	0.004	126	5	0	95	2	0	Yes
	1:22903028	rs370843084	G	A		missense	Gly160Ser	0.00003	-	131	0	0	96	1	0	Yes
	1:22913111	rs56656925	C	T		missense	Pro321Leu	0.00035	0.003	130	1	0	97	0	0	Yes
	1:22915462	rs762631777	A	G		missense	Ile360Val	0.00002	-	131	0	0	96	1	0	Yes
	1:22915477	rs369589341	G	A		missense	Val365Met	0.00011	-	130	1	0	96	1	0	Yes
	1:22919833	rs2295021	G	A		missense	Val444Met	0.00000	-	130	1	0	97	0	0	Yes
	1:22919888	rs77608596	A	G		missense	Glu462Gly	0.00150	-	130	1	0	96	1	0	Yes
	1:22919894	rs777499719	A	G		missense	Glu464Gly	0.00000	-	130	1	0	97	0	0	Yes
	1:22920150	rs149084883	A	G		missense	Gln525Arg	0.00308	0.002	131	0	0	96	1	0	Yes

	1:22921794	rs200214765	C	T		missense	Leu559Phe	0.00088	0.001	130	1	0	97	0	0	Yes
	1:22923859	rs144329757	C	A		missense	Pro607His	0.00844	0.004	128	3	0	97	0	0	Yes
	1:22923861	-	C	T		**stop gained**	Gln608*	-	-	130	1	0	97	0	0	Yes
	1:22923873	rs999765	G	C		missense	Glu612Gln	0.10359	0.078	109	20	2	82	11	4	Yes
	1:22924274	rs562829959	G	A		missense	Arg679Gln	0.00000	-	131	0	0	96	1	0	Yes
	1:22925524	-	C	T		missense	Ala791Val	-	-	130	1	0	97	0	0	Yes
	1:22927503	rs62618734	G	A		missense	Arg884His	0.00930	0.006	129	2	0	95	2	0	Yes
	1:22927881	rs374945795	G	C		missense	Asp940His	0.00000	-	130	1	0	97	0	0	Yes
FAM134B (ENSG00000154153)	5:16475199	rs61733811	C	G	ENST00000306320	missense	Ser382Thr	0.00070	0.004	130	1	0	96	1	0	Yes
	5:16475209	rs34432513	G	C		missense	Gln379Glu	0.00015	0.004	131	0	0	96	1	0	Yes
	5:16481181	rs143878016	C	T		missense	Val203Met	0.00425	-	131	0	0	96	1	0	Yes
	5:16483487	rs756538225	T	C		missense	Met185Val	0.00002	-	131	0	0	96	1	0	Yes
	5:16483574	rs758377163	C	A		missense	Val156Phe	0.00002	-	130	1	0	97	0	0	Yes
	5:16572153	rs78314670	G	A		missense	Arg127Cys	0.01020	0.004	130	1	0	93	4	0	Yes
	5:16617064	-	G	A		missense	Pro6Leu	-	-	130	0	1	97	0	0	Yes
FGD4 (ENSG00000139132)	12:32735037	rs145115430	C	T	ENST00000427716	missense	Thr79Ile	0.00006	0.002	130	1	0	97	0	0	Yes
	12:32754345	rs756169087	G	A		missense	Arg275Gln	0.00002	-	131	0	0	96	1	0	Yes
	12:32763752	rs781528826	G	C		missense	Ser392Thr	-	-	130	1	0	97	0	0	Yes
	12:32772675	-	T	C		missense	Val461Ala	-	-	131	0	0	96	1	0	Yes
	12:32777929	rs141237776	A	G		missense	Asp521Gly	0.00013	-	131	0	0	96	1	0	Yes
	12:32778663	rs144693221	C	A		missense	Pro571Thr	0.00619	0.002	130	1	0	96	1	0	Yes
	12:32793315	rs61753359	G	A		missense	Val717Met	0.00189	0.001	130	1	0	97	0	0	Yes
FIG4 (ENSG00000112367)	6:110036336	rs121908287	T	C	ENST00000230124	missense	Ile41Thr	0.00155	0.001	129	2	0	97	0	0	Yes
	6:110036365	-	A	G		missense	Ile51Val	-	-	131	0	0	96	1	0	Yes
	6:110062705	rs138048706	A	T		missense	Lys278Asn	0.00049	0.001	130	1	0	97	0	0	Yes
	6:110064928	rs2295837	A	T		missense	Met364Leu	0.03636	0.038	123	8	0	92	5	0	Yes
	6:110081504	-	G	C		missense	Ala397Pro	-	-	131	0	0	96	1	0	Yes
	6:110107517	rs9885672	T	C		missense	Val654Ala	0.15789	0.148	92	34	5	74	23	0	Yes
	6:110112598	rs372846619	G	A		missense	Glu734Lys	0.00014	-	131	0	0	96	1	0	Yes
GARS (ENSG00000106105)	7:30634548	rs62636572	C	T	ENST00000389266	missense	Pro4Leu	0.02812	0.003	126	5	0	94	2	1	Yes
	7:30634658	rs762605231	T	C		missense	Cys41Arg	0.00000	-	130	1	0	97	0	0	Yes
	7:30634661	rs1049402	C	G		missense	Pro42Ala	0.76849	0.261	25	31	75	20	21	56	No

	7:30638491	rs200887429	G	A		missense	Arg101His	0.00061	0.001	130	1	0	97	0	0	Yes
	7:30649268	rs2230310	C	T		missense	Thr268Ile	0.00475	0.005	131	0	0	92	5	0	Yes
	7:30661058	-	C	T		missense	Ser470Phe	-	-	130	1	0	97	0	0	Yes
	7:30668236	rs750292154	C	T		missense	Thr587Met	0.00002	-	130	1	0	97	0	0	Yes
HSPB1 (ENSG00000106211)	7:75932237	-	G	A	ENST00000248553	missense	Ala70Thr	-	-	131	0	0	96	1	0	Yes
HSPB8 (ENSG00000152137)	12:119617202	rs748320300	C	T	ENST00000281938	missense	Arg29Cys	0.00002	-	131	0	0	96	1	0	Yes
IKBKAP (ENSG00000070061)	9:111641825	rs1538660	G	A	ENST00000374647	missense	Pro1158Leu	0.17074	0.166	101	28	2	68	26	3	Yes
	9:111651620	rs3204145	A	T		missense	Cys1072Ser	0.17022	0.181	96	33	2	65	29	3	Yes
	9:111653606	rs2230795	C	T		missense	Gly1013Ser	0.00013	0.001	130	1	0	97	0	0	Yes
	9:111656228	rs2230798	T	A		missense	Lys952Ile	0.01692	0.008	130	1	0	95	2	0	Yes
	9:111659439	rs2230794	T	C		missense	Ile830Met	0.04604	0.057	114	17	0	91	6	0	Yes
	9:111659483	rs2230793	T	G		missense	Ile816Leu	0.18077	0.211	81	42	8	60	34	3	Yes
	9:111660851	rs2230792	C	T		missense	Gly765Glu	0.18670	0.207	81	42	8	60	34	3	Yes
	9:111663930	rs148378319	C	T		missense	Arg629His	0.00277	0.002	130	1	0	96	1	0	Yes
	9:111668652	rs838827	C	T		missense	Arg525Gln	0.06139	0.089	113	14	4	84	12	1	Yes
	9:111674716	rs56053149	G	T		missense	Ser339Arg	0.00004	-	131	0	0	96	1	0	Yes
	9:111678508	rs1140064	C	T		missense	Glu312Lys	0.02805	0.017	128	3	0	95	2	0	Yes
	9:111679940	rs17853166	T	C		missense	Ser251Gly	0.02803	0.016	129	2	0	95	2	0	Yes
	9:111685129	rs10521092	A	T		missense	Met182Lys	0.00018	0.002	130	1	0	96	1	0	Yes
	9:111693276	-	C	T		**splice donor**	c.150+1G>A	-	-	131	0	0	96	1	0	Yes
KIF1B (ENSG00000054523)	1:10292415	-	T	C	ENST00000263934	missense	Val10Ala	-	-	130	1	0	97	0	0	Yes
	1:10381802	rs551543997	T	C		missense	Trp703Arg	0.00009	-	131	0	0	96	1	0	Yes
	1:10397567	rs2297881	A	G		missense	Tyr1087Cys	0.02518	-	121	10	0	87	10	0	Yes
	1:10425683	rs779756425	C	G		missense	Arg1531Gly	0.00000	-	130	1	0	97	0	0	Yes
	1:10428570	rs77172218	G	A		missense	Val1554Met	0.01600	-	129	2	0	96	1	0	Yes
LITAF (ENSG00000189067)	16:11643394	rs149712652	G	A	ENST00000571688	missense	Pro196Ser	0.01258	0.011	128	3	0	96	1	0	Yes
	16:11647492	rs4280262	T	C		missense	Ile92Val	0.20581	0.164	83	43	5	61	31	5	Yes
	16:11650441	rs141862602	G	A		missense	Thr49Met	0.00077	0.003	130	1	0	96	1	0	Yes
	16:11650487	rs759905004	G	T		missense	Pro34Thr	0.00002	-	130	1	0	97	0	0	Yes

LRSAM1 (ENSG00000148356)	9:130230038	rs75690855	C	T	ENST00000323301	missense	Ser183Leu	0.00047	0.001	131	0	0	96	1	0	Yes
	9:130236144	rs376671005	C	G		missense	Ile228Met	0.00002	-	130	1	0	97	0	0	Yes
	9:130241219	rs762870327	T	G		missense	Phe253Val	-	0.001	130	1	0	97	0	0	Yes
	9:130242166	rs1539567	A	G		missense	Asn318Asp	0.78166	0.343	18	47	66	10	37	50	Yes
	9:130248080	rs149540339	C	G		missense	Gln409Glu	0.00078	-	130	1	0	97	0	0	Yes
	9:130253569	rs749192098	C	T		missense	Leu500Phe	0.00006	-	130	1	0	97	0	0	Yes
	9:130258261	rs150882646	C	A		missense	Gln573Lys	0.00017	-	130	1	0	97	0	0	Yes
	9:130258324	rs150062009	C	T		missense	Arg594Cys	0.00033	-	131	0	0	96	1	0	Yes
	9:130263292	rs745672498	T	C		missense	Leu639Pro	0.00002	-	130	1	0	97	0	0	Yes
	9:130265096	-	A	G		missense	Gln697Arg	-	-	131	0	0	96	1	0	Yes
MED25 (ENSG00000104973)	19:50321695	-	G	C	ENST00000312865	missense	Glu33Gln	-	-	131	0	0	96	1	0	Yes
	19:50334047	rs145770066	C	T		missense	Ala335Val	0.00604	-	131	0	0	95	2	0	Yes
	19:50338843	rs193291405	C	G		missense	Ala576Gly	0.02446	0.002	130	1	0	96	1	0	Yes
MFN2 (ENSG00000116688)	1:12052623	rs761216583	A	C	ENST00000235329	missense	Asn63His	0.00003	-	130	1	0	97	0	0	Yes
	1:12061533	rs41278630	G	A		missense	Gly298Arg	0.00316	0.005	130	1	0	97	0	0	Yes
	1:12064155	rs765921889	A	G		missense	Thr423Ala	0.00000	-	130	1	0	97	0	0	Yes
	1:12064892	rs138382758	G	A		missense	Arg468His	0.00325	0.009	129	2	0	96	1	0	Yes
	1:12067224	rs369762154	C	T		missense	Arg663Cys	0.00012	-	130	1	0	97	0	0	Yes
	1:12069692	rs142271930	G	A		missense	Val705Ile	0.00628	0.002	130	1	0	96	1	0	Yes
	1:12069698	rs119103267	C	T		missense	Arg707Trp	0.00055	0.001	131	0	0	96	1	0	Yes
MTMR2 (ENSG00000087053)	11:95568531	rs116750638	A	G	ENST00000346299	missense	Ser619Pro	0.00255	0.002	130	1	0	96	1	0	Yes
	11:95571347	rs61735578	C	G		missense	Glu502Gln	0.02432	0.023	127	4	0	95	2	0	Yes
	11:95578167	rs146572467	C	T		missense	Glu446Lys	0.00055	0.002	130	1	0	97	0	0	Yes
	11:95590766	rs186380748	G	C		missense	Pro202Ala	0.00047	0.002	129	2	0	97	0	0	Yes
	11:95657111	rs3824874	T	G		missense	Lys3Thr	0.42441	0.222	53	51	27	49	30	18	No
NDRG1 (ENSG00000104419)	8:134251197	rs367925853	G	A	ENST00000414097	missense	Ala370Val	0.00052	-	131	0	0	96	1	0	Yes
	8:134251215	rs767058269	G	C		missense	Ser364Trp	-	-	131	0	0	96	1	0	Yes
	8:134296524	rs145871479	C	T		missense	Ala11Thr	0.00139	0.004	131	0	0	94	3	0	Yes
NGF (ENSG00000134259)	1:115829178	rs11466111	C	T	ENST00000369512	missense	Arg80Gln	0.01634	0.008	127	4	0	95	2	0	Yes
	1:115829313	rs6330	G	A		missense	Ala35Val	0.44519	0.443	47	62	22	32	44	21	Yes
	1:156830779	rs1007211	G	A	ENST00000524377	missense	Gly18Glu	0.01562	0.002	130	1	0	96	1	0	Yes
	1:156834169	rs139140006	T	A		missense	Leu79Gln	0.00006	-	130	1	0	97	0	0	Yes

NTRK1 (ENSG00000198400)	1:156838297	rs201185829	G	C		missense	Gly192Ala	0.00133	-	130	1	0	97	0	0	Yes
	1:156841437	-	T	C		missense	Leu247Pro	-	-	131	0	0	96	1	0	Yes
	1:156846268	-	A	G		missense	Gln570Arg	-	-	131	0	0	96	1	0	Yes
	1:156848918	rs6336	C	T		missense	His604Tyr	0.05491	0.046	120	10	1	89	8	0	Yes
	1:156848946	rs6339	G	T		missense	Gly613Val	0.05483	0.047	120	10	1	89	8	0	Yes
	1:156849884	rs770727871	G	A		missense	Gly714Ser	0.00002	-	131	0	0	96	1	0	Yes
	1:156851379	-	C	G		missense	Ala779Gly	-	-	131	0	0	96	1	0	Yes
	1:156851382	rs35669708	G	A		missense	Arg780Gln	0.00722	0.003	129	2	0	93	4	0	Yes
PMP22 (ENSG00000109099)	17:15134308	rs755551524	T	C	ENST00000395938	missense	Ile137Val	0.00000	-	130	1	0	96	1	0	Yes
	17:15134364	rs104894619	G	A		missense	Thr118Met	0.00736	0.003	123	0	0	83	1	0	Yes
PRX (ENSG00000105227)	19:40900077	-	C	A	ENST00000324001	missense	Glu1394Asp	-	-	131	0	0	96	1	0	Yes
	19:40900179	-	TTCC	T		inframe del	Glu1360del	-	-	131	0	0	96	1	0	Yes
	19:40900490	rs200332462	C	T		missense	Gly1257Arg	0.00002	0.002	131	0	0	96	1	0	Yes
	19:40900865	rs268674	C	T		missense	Gly1132Arg	0.94233	0.279	2	16	113	0	4	93	Yes
	19:40901011	rs3745202	G	C		missense	Pro1083Arg	0.17537	0.160	124	4	3	89	6	2	No
	19:40901073	rs139188673	C	A		missense	Lys1062Asn	0.00152	0.006	131	0	0	96	1	0	Yes
	19:40901222	rs548086012	C	T		missense	Asp1013Asn	0.00003	0.001	130	1	0	97	0	0	Yes
	19:40901496	rs268673	T	C		missense	Ile921Met	0.39123	0.350	46	61	24	33	49	15	Yes
	19:40901614	rs268671	A	G		missense	Val882Ala	0.51248	0.379	28	63	40	27	48	22	Yes
	19:40901647	rs201389706	A	G		missense	Val871Ala	0.04189	0.003	116	15	0	87	10	0	Yes
	19:40901992	rs749585237	G	A		missense	Pro756Leu	0.00006	-	130	1	0	97	0	0	Yes
	19:40902008	-	A	G		missense	Ser751Pro	-	-	131	0	0	96	1	0	Yes
	19:40902251	rs757467172	T	C		missense	Met670Val	-	-	130	1	0	96	1	0	Yes
	19:40902776	rs146789340	C	G		missense	Glu495Gln	0.00393	0.011	130	1	0	97	0	0	Yes
	19:40903528	rs118071705	G	A		missense	Ala244Val	0.03438	0.004	129	2	0	94	3	0	Yes
SBF2 (ENSG00000133812)	11:9801969	-	G	A	ENST00000256190	missense	Ala1849Val	-	-	131	0	0	96	1	0	Yes
	11:9809201	-	CTTT	C		inframe del	Lys1672del	0.00061	-	130	1	0	97	0	0	Yes
	11:9830504	rs758191255	C	T		missense	Glu1401Lys	-	-	130	1	0	97	0	0	Yes
	11:9850939	-	T	A		missense	Thr1253Ser	-	-	131	0	0	96	1	0	Yes
	11:9853777	rs12574508	G	C		missense	Gln1216Glu	0.10937	0.086	106	23	2	76	18	3	Yes
	11:9861208	rs117957652	G	C		missense	Leu1098Val	0.02703	0.025	128	3	0	92	5	0	Yes

	11:9871708	-	G	C		missense	Arg890Gly	-	-	130	1	0	97	0	0	Yes
	11:9878045	rs141330687	C	T		missense	Gly775Ser	0.00409	0.002	130	1	0	97	0	0	Yes
	11:9878180	-	A	C		missense	Ser730Ala	-	-	130	1	0	97	0	0	Yes
	11:9879838	rs7102464	C	T		missense	Glu679Lys	0.10425	0.102	107	24	0	78	19	0	Yes
	11:10015505	rs149794117	G	A		missense	Pro339Leu	0.00003	-	130	1	0	97	0	0	Yes
	11:10019878	-	C	T		missense	Glu304Lys	0.00002	-	130	1	0	97	0	0	Yes
	11:10019921	rs775319050	A	C		missense	Asp289Glu	0.00000	-	131	0	0	96	1	0	Yes
SCN9A (ENSG00000169432)	2:167055370	rs111558968	G	A	ENST00000409672	missense	Leu1916Phe	0.00015	0.002	130	1	0	97	0	0	Yes
	2:167055393	rs3750904	T	C		missense	Asp1908Gly	0.00320	0.011	131	0	0	96	1	0	Yes
	2:167083160	rs149346064	C	T		missense	Val1428Ile	0.00286	0.001	130	1	0	97	0	0	Yes
	2:167089942	rs180922748	G	C		missense	Leu1267Val	0.00220	0.006	131	0	0	96	1	0	Yes
	2:167094638	rs141268327	T	C		missense	Asn1245Ser	0.00809	0.012	127	4	0	95	2	0	Yes
	2:167094715	rs750397053	G	C		missense	Asp1219Glu	0.00000	0.002	131	0	0	96	1	0	Yes
	2:167099158	rs6746030	A	G		missense	Trp1150Arg	0.87293	0.215	19	17	95	17	22	58	No
	2:167108385	rs74401238	C	T		missense	Arg1110Gln	0.02084	0.012	130	1	0	95	2	0	Yes
	2:167136962	rs182650126	T	C		missense	Ile739Val	0.00626	0.004	130	1	0	96	1	0	Yes
	2:167138296	rs121908919	T	C		missense	Lys655Arg	0.00290	0.008	130	1	0	96	1	0	Yes
	2:167141109	rs41268673	G	T		missense	Pro610Thr	0.03443	0.029	119	12	0	91	6	0	Yes
	2:167142979	rs58022607	C	T		missense	Ser490Asn	0.00546	0.008	130	1	0	94	3	0	Yes
	2:167163032	rs761441210	G	T		missense	Thr152Asn	-	-	131	0	0	96	1	0	Yes
	2:167168046	rs201992546	G	T		missense	Pro74His	-	-	131	0	0	96	1	0	Yes
	2:167168149	rs371565974	T	C		missense	Lys40Glu	0.00004	0.001	130	1	0	97	0	0	Yes
SEPT9 (ENSG00000184640)	17:75398351	-	C	T	ENST00000427177	missense	Ser96Leu	-	-	130	1	0	97	0	0	Yes
	17:75398498	rs34587622	C	T		missense	Pro145Leu	0.12237	0.130	90	33	8	71	26	0	Yes
	17:75398768	rs528907798	C	T		missense	Thr235Ile	0.00000	-	130	1	0	97	0	0	Yes
	17:75483634	rs201560726	G	A		missense	Asp348Asn	0.00021	-	130	1	0	97	0	0	Yes
	17:75484342	rs199557573	C	T		missense	Arg355Trp	0.00055	-	130	1	0	97	0	0	Yes
	17:75494705	rs2627223	A	G		missense	Met576Val	0.93855	0.215	38	8	85	21	3	73	No
SH3TC2 (ENSG00000169247)	5:148384455	rs146920285	T	A	ENST00000515425	missense	Asp1229Val	0.00367	0.006	128	3	0	96	1	0	Yes
	5:148388420	rs55853803	C	T		missense	Val1158Ile	0.03139	0.029	123	8	0	93	4	0	Yes
	5:148389868	rs77636085	T	G		missense	Thr1098Pro	0.00188	-	130	1	0	96	1	0	Yes

	5:148406803	rs375034766	C	T		missense	Ser831Asn	0.00007	-	130	1	0	97	0	0	Yes
	5:148407031	-	G	A		missense	Thr755Ile	-	-	130	1	0	97	0	0	Yes
	5:148407208	rs17109261	T	C		missense	His696Arg	0.00013	0.003	130	1	0	97	0	0	Yes
	5:148407322	rs138040787	C	T		missense	Arg658His	0.00009	-	130	1	0	97	0	0	Yes
	5:148407527	rs149244124	C	T		missense	Ala590Thr	0.00004	-	130	1	0	97	0	0	Yes
	5:148407766	rs757294130	T	G		missense	Tyr510Ser	0.00000	-	130	1	0	97	0	0	Yes
	5:148407893	rs6875902	C	A		missense	Ala468Ser	0.21292	0.210	78	49	4	67	27	3	Yes
	5:148407997	rs200967041	G	A		missense	Ser433Leu	0.00074	-	130	1	0	97	0	0	Yes
	5:148411156	rs772832716	T	C		missense	Thr366Ala	0.00003	-	130	1	0	97	0	0	Yes
	5:148420221	rs144963732	G	A		missense	Pro251Ser	0.00027	-	131	0	0	96	1	0	Yes
	5:148421021	rs148634904	A	G		missense	Val230Ala	0.00081	0.003	130	1	0	96	1	0	Yes
	5:148422274	rs17722293	C	T		missense	Gly171Glu	0.01443	0.020	128	3	0	95	2	0	Yes
	5:148431777	rs141649676	T	C		missense	Thr27Ala	0.00167	-	130	1	0	97	0	0	Yes
SLCO1B1 (ENSG00000134538)	12:21294537	rs766950888	C	T	ENST00000256958	missense	Thr10Ile	0.00002	-	130	1	0	97	0	0	Yes
	12:21329738	rs2306283	A	G		missense	Asn130Asp	0.41084	0.384	49	60	22	33	40	24	Yes
	12:21329813	rs11045819	C	A		missense	Pro155Thr	0.16647	0.142	102	27	2	68	27	2	Yes
	12:21331549	rs4149056	T	C		missense	Val174Ala	0.16052	0.124	96	29	6	70	27	0	Yes
	12:21331605	rs376996580	C	A		missense	Leu193Ile	0.00000	-	130	1	0	95	2	0	Yes
	12:21331856	rs766417954	G	T		missense	Gly210Val	0.00000	-	131	0	0	96	1	0	Yes
	12:21375289	rs71581941	C	T		**stop gained**	Arg580*	0.00141	0.002	130	1	0	97	0	0	Yes
	12:21391976	rs34671512	A	C		missense	Leu643Phe	0.05201	0.054	119	11	1	84	12	1	Yes
	12:21392013	rs757219127	A	G		missense	Ile656Val	0.00002	-	130	1	0	97	0	0	Yes
SLCO1B3 (ENSG00000111700)	12:20968739	rs369736559	C	T	ENST00000381545	missense	Arg23Cys	0.00011	-	130	1	0	97	0	0	Yes
	12:21007985	rs79042365	C	G		missense	Phe36Leu	0.00000	-	131	0	0	96	1	0	Yes
	12:21008067	rs151295214	T	A		missense	Ser64Thr	0.00000	-	130	1	0	97	0	0	Yes
	12:21011480	rs4149117	T	G		missense	Ser112Ala	0.85331	0.190	6	27	98	4	20	73	Yes
	12:21014025	rs146623116	A	G		missense	Asn145Ser	0.00248	0.002	129	2	0	96	1	0	Yes
	12:21015760	rs7311358	G	A		missense	Met233Ile	0.85329	0.104	2	27	102	1	22	74	Yes
	12:21015764	-	G	A		missense	Val235Met	-	-	130	1	0	97	0	0	Yes
	12:21028208	rs60140950	G	C		missense	Gly256Ala	0.16225	0.170	101	27	3	69	25	3	Yes
	12:21032475	rs146940490	C	T		missense	Thr414Ile	0.00045	0.001	131	0	0	96	1	0	Yes

SPTLC1 (ENSG00000090054)	9:94800624	rs119482084	C	G	ENST00000262554	missense	Gly387Ala	0.00050	0.004	129	2	0	97	0	0	Yes
	9:94830356	rs45461899	C	A		missense	Arg151Leu	0.03106	0.021	127	4	0	94	3	0	Yes
	9:94830357	rs146548058	G	A		missense	Arg151Cys	0.00003	-	130	1	0	97	0	0	Yes
	9:94843240	rs76962472	G	T		missense	Pro89Gln	0.00063	-	131	0	0	96	1	0	Yes
SPTLC2 (ENSG00000100596)	14:77987874	rs747168398	G	A	ENST00000216484	missense	Arg452Cys	0.00002	-	131	0	0	96	1	0	Yes
TRPV4 (ENSG00000111199)	12:110221524	rs55728855	C	T	ENST00000418703	missense	Glu840Lys	0.00885	0.004	129	2	0	97	0	0	Yes
	12:110221571	rs764622721	G	A		missense	Ser824Leu	0.00005	-	131	0	0	96	1	0	Yes
	12:110230597	rs56177950	C	T		missense	Val562Ile	0.01019	0.015	128	3	0	93	4	0	Yes
	12:110231809	rs762000967	T	C		missense	Thr504Ala	0.00000	-	131	0	0	96	1	0	Yes
	12:110234491	rs775385702	G	A		missense	Arg391Trp	0.00000	-	131	0	0	96	1	0	Yes
	12:110236693	-	G	T		missense	Ala293Asp	-	-	130	1	0	97	0	0	Yes
	12:110246181	rs139300843	C	T		missense	Arg160Gln	0.00003	-	131	0	0	96	1	0	Yes
	12:110252547	rs3742030	G	A		missense	Pro19Ser	0.03914	0.023	127	4	0	89	7	1	Yes

[a]MAF in ExAC for Europeans non-Finish (>33.000 indiv).

[b]MAF in Spanish population (578 indiv).

Supplementary Table 2. Association of common non-synonymous coding variants with paclitaxel-induced neuropathy.

Gene	Variant ID	Protein change	MAF ExAC[a]	MAF Spanish[b]	Nr carriers		OR, 95% CI (inf-sup)	P value
					High neuropathy	No/low neuropathy		
AARS	rs35744709	Lys967Met	0.01467	0.014	7	4	1.30 (0.33-6.16)	0.766
	rs149377346	Gly931Ser	0.01102	0.010	3	3	0.74 (0.10-5.57)	0.703
	rs147319762	Lys820Arg	0.00182	0.014	2	3	0.49 (0.04-4.33)	0.655
ABCB1	rs55852620	Gln1107Pro	0.00582	0.002	2	0	1.01 (0.99-1.02)	0.510
	rs2229109	Ser400Asn	0.04316	0.023	7	10	0.51 (0.16-1.50)	0.257
	rs9282564	Asn21Asp	0.11199	0.053	19	17	0.76 (0.37-1.59)	0.542
ARHGEF10	rs9657362	Leu370Phe	0.13846	0.119	21	26	0.62 (0.33-1.18)	0.155
	rs2294039	Val700Ile	0.04063	0.055	8	9	0.66 (0.23-1.83)	0.502
	rs61752020	Val938Ile	0.00817	0.011	2	3	0.49 (0.04-4.33)	0.655
	rs17683288	Ser984Ala	0.07288	0.057	20	10	1.52 (0.66-3.73)	0.387
CCT5	rs11557652	Glu146Val	0.02393	0.012	3	2	1.11 (0.13-13.44)	1.000
	rs141675330	Ile362Met	0.00511	0.004	1	1	0.74 (0.01-58.32)	1.000
CYP2C8	rs10509681	Lys399Arg	0.11299	0.152	44	33	0.98 (0.59-1.62)	1.000
	rs1058930	Ile264Met	0.05429	0.059	15	21	0.45 (0.21-0.93)	**0.030**
	rs41286886	Val181Ile	0.00762	0.001	1	1	0.74 (0.01-58.32)	1.000
	rs11572080	Arg139Lys	0.11287	0.137	39	30	0.96 (0.57-1.63)	0.966
DHTKD1	rs34644609	Ala70Gly	0.00684	0.008	3	1	2.23 (0.18-117.89)	0.640
	rs3740015	Tyr272Asp	0.59906	0.427	76	70	0.80 (0.54-1.18)	0.275
	rs147571909	Val360Ala	0.00558	0.003	1	1	0.74 (0.01-58.32)	1.000
	rs2062988	Ile607Met	0.81243	0.168	46	34	1.03 (0.62-1.70)	1.000
EPHA5	rs36050417	Ala672Thr	0.02758	0.038	7	6	0.86 (0.24-3.15)	1.000
	rs33932471	Asn81Thr	0.06647	0.065	16	9	1.34 (0.54-3.51)	0.636
EPHA6	rs4857276	Ala805Val	0.00742	0.011	2	0	1.01 (0.99-1.02)	0.510
EPHA8	rs45498698	Gly45Ser	0.00821	0.004	5	2	1.87 (0.30-19.78)	0.704
	rs144329757	Pro607His	0.00844	0.004	3	0	1.01 (0.99-1.03)	0.265
	rs999765	Glu612Gln	0.10359	0.078	22	15	0.93 (0.47-1.85)	0.947
	rs62618734	Arg884His	0.00930	0.006	2	2	0.74 (0.05-10.28)	1.000
FAM134B	rs78314670	Arg127Cys	0.01020	0.004	1	4	0.18 (0.00-1.86)	0.168

FGD4	rs144693221	Pro571Thr	0.00619	0.002	1	1	0.74 (0.01-58.32)	1.000
FIG4	rs2295837	Met364Leu	0.03636	0.038	8	5	1.19 (0.34-4.70)	0.986
	rs9885672	Val654Ala	0.15789	0.148	39	23	1.50 (0.85-2.71)	0.181
GARS	rs62636572	Pro4Leu	0.02812	0.003	5	3	0.92 (0.20-4.72)	1.000
IKBKAP	rs1538660	Pro1158Leu	0.17074	0.166	30	29	0.70 (0.40-1.24)	0.244
	rs3204145	Cys1072Ser	0.17022	0.181	35	32	0.75 (0.44-1.28)	0.315
	rs2230798	Lys952Ile	0.01692	0.008	1	2	0.37 (0.01-7.13)	0.577
	rs2230794	Ile830Met	0.04604	0.057	17	6	2.17 (0.80-6.86)	0.155
	rs2230793	Ile816Leu	0.18077	0.211	50	37	1.09 (0.68-1.77)	0.783
	rs2230792	Gly765Glu	0.18670	0.207	50	37	1.09 (0.68-1.77)	0.783
	rs838827	Arg525Gln	0.06139	0.089	18	13	1.18 (0.56-2.56)	0.774
	rs1140064	Glu312Lys	0.02805	0.017	3	2	1.11 (0.13-13.44)	1.000
	rs17853166	Ser251Gly	0.02803	0.016	2	2	0.74 (0.05-10.28)	1.000
KIF1B	rs2297881	Tyr1087Cys	0.02518	-	10	10	0.73 (0.27-2.00)	0.647
	rs77172218	Val1554Met	0.01600	-	2	1	1.48 (0.08-87.99)	1.000
LITAF	rs149712652	Pro196Ser	0.01258	0.011	3	1	2.23 (0.18-117.89)	0.640
	rs4280262	Ile92Val	0.20581	0.164	48	36	0.95 (0.58-1.54)	0.905
LRSAM1	rs1539567	Asn318Asp	0.78166	0.343	65	47	1.11 (0.73-1.71)	0.672
MED25	rs145770066	Ala335Val	0.00604	-	0	2	0.00 (0.00-3.94)	0.180
	rs193291405	Ala576Gly	0.02446	0.002	1	1	0.74 (0.01-58.32)	1.000
MFN2	rs142271930	Val705Ile	0.00628	0.002	1	1	0.74 (0.01-58.32)	1.000
MTMR2	rs61735578	Glu502Gln	0.02432	0.023	4	2	1.49 (0.21-16.61)	1.000
NGF	rs11466111	Arg80Gln	0.01634	0.008	4	2	1.49 (0.21-16.61)	1.000
	rs6330	Ala35Val	0.44519	0.443	84	65	0.85 (0.58-1.26)	0.464
NTRK1	rs1007211	Gly18Glu	0.01562	0.002	1	1	0.74 (0.01-58.32)	1.000
	rs6336	His604Tyr	0.05491	0.046	11	8	1.12 (0.41-3.21)	0.997
	rs6339	Gly613Val	0.05483	0.047	11	8	1.12 (0.41-3.21)	0.997
	rs35669708	Arg780Gln	0.00722	0.003	2	4	0.37 (0.03-2.58)	0.409
PMP22	rs104894619	Thr118Met	0.00736	0.003	0	1	0.00 (0.00-28.88)	0.425
PRX	rs268674	Gly1132Arg	0.94233	0.279	18	4	3.92 (1.28-16.01)	**0.015**
	rs268673	Ile921Met	0.39123	0.350	85	64	1.04 (0.70-1.54)	0.926
	rs268671	Val882Ala	0.51248	0.379	103	70	1.33 (0.90-1.97)	0.156
	rs201389706	Val871Ala	0.04189	0.003	15	10	1.12 (0.46-2.85)	0.955
	rs146789340	Glu495Gln	0.00393	0.011	1	0	1.00(0.99-1.01)	1.000

	rs118071705	Ala244Val	0.03438	0.004	2	3	0.49 (0.04-4.33)	0.655
SBF2	rs12574508	Gln1216Glu	0.10937	0.086	25	21	0.81 (0.44-1.53)	0.588
	rs117957652	Leu1098Val	0.02703	0.025	3	5	0.44 (0.07-2.29)	0.294
	rs7102464	Glu679Lys	0.10425	0.102	24	19	0.93 (0.47-1.85)	0.947
SCN9A	rs141268327	Asn1245Ser	0.00809	0.012	4	2	1.49 (0.21-16.61)	1.000
	rs74401238	Arg1110Gln	0.02084	0.012	1	2	0.37 (0.01-7.13)	0.577
	rs182650126	Ile739Val	0.00626	0.004	1	1	0.74 (0.01-58.32)	1.000
	rs41268673	Pro610Thr	0.03443	0.029	12	6	1.50 (0.51-4.97)	0.573
	rs58022607	Ser490Asn	0.00546	0.008	1	3	0.24 (0.00-3.07)	0.317
SEPT9	rs34587622	Pro145Leu	0.12237	0.130	41	26	1.49 (0.86-2.60)	0.167
SH3TC2	rs55853803	Val1158Ile	0.03139	0.029	8	4	1.49 (0.39-6.88)	0.720
	rs6875902	Ala468Ser	0.21292	0.210	53	30	1.36 (0.82-2.26)	0.254
	rs17722293	Gly171Glu	0.01443	0.020	3	2	1.11 (0.13-13.44)	1.000
SLCO1B1	rs2306283	Asn130Asp	0.41084	0.384	82	64	0.79 (0.54-1.18)	0.265
	rs11045819	Pro155Thr	0.16647	0.142	29	29	0.71 (0.40-1.25)	0.255
	rs4149056	Val174Ala	0.16052	0.124	35	27	1.15 (0.66-2.02)	0.704
	rs34671512	Leu643Phe	0.05201	0.054	12	13	0.67 (0.28-1.58)	0.419
SLCO1B3	rs4149117	Ser112Ala	0.85331	0.190	33	24	1.04 (0.59-1.83)	0.999
	rs7311358	Met233Ile	0.85329	0.104	29	23	0.95 (0.52-1.76)	0.977
	rs60140950	Gly256Ala	0.16225	0.170	30	28	0.76 (0.43-1.34)	0.372
SPTLC1	rs45461899	Arg151Leu	0.03106	0.021	4	3	0.99 (0.16-6.82)	1.000
TRPV4	rs55728855	Glu840Lys	0.00885	0.004	2	0	1.01 (0.99-1.02)	0.510
	rs56177950	Val562Ile	0.01019	0.015	3	4	0.55 (0.08-3.30)	0.465
	rs3742030	Pro19Ser	0.03914	0.023	4	8	0.32 (0.07-1.17)	0.091

[a]MAF in ExAC for Europeans non-Finish (>33.000 indiv).

[b]MAF in Spanish population (578 indiv).

DISCUSSION

Thanks to the advances in oncology treatment, the number of cancer survivors is increasing. Despite this, anticancer therapy causes a high number of serious ADRs, some of which can have a great impact on the quality of life of the cancer patients. One example is the antimitotic agent paclitaxel (Wani et al., 1971, Rowinsky, 1997), widely used since the late twentieth century. It produces peripheral neuropathy (Rowinsky et al., 1993) in a large number of patients and, in some cases, with long-term nerve damage and long-life disabilities (Postma et al., 1995, Lipton et al., 1989). There is a lack of preventive or effective treatments (Wolf et al., 2008) and, therefore, the identification of neuropathy markers is an urgent need, to guide taxanes treatment and avoid serious and potentially permanent neurotoxicity.

The individual genetic background has been shown to explain a great proportion of the inter-individual variability observed in the response to many different drugs; however, the majority of genetic factors related to paclitaxel-induced neuropathy remain undetermined. Although several groups have evaluated the role of common polymorphisms in genes involved in paclitaxel metabolism and transport (e.g. *CYP2C8*3*), their contribution to neuropathy is unclear. Moreover, only a few studies have been conducted to analyze the relevance of variants across the whole genome or to detect low-frequency variants associated with the neuropathy.

Owing to these, the purpose of this Thesis was to identify genetic variants associated with increased susceptibility of peripheral neuropathy caused by paclitaxel. To accomplish this goal, series of hundreds of patients treated with paclitaxel and with complete neuropathy data were collected. To ensure homogeneity and objectivity in neuropathy grading, all patients were interviewed by telephone by one qualified nurse trained by a neurologist to determine the severity of symptoms and impairment in the activities of daily living during paclitaxel treatment and after finishing therapy. The neuropathy assessment was performed according to NCI-CTCAE v4. In addition, part of the patients also filled in a questionnaire after each paclitaxel dose and thus, cycle by cycle neuropathy data was available. In addition, neuropathy risk factors such as previous neurotoxic treatments, presence of diabetes or alcoholism were also recorded.

In the first part of this Thesis we performed a comprehensive study of common single nucleotide polymorphisms previously associated with paclitaxel-induced neuropathy by our group, and investigated their role as potential predictive markers of this toxicity (Article 1 and Article 2).

1. Role of *CYP2C8*3* in paclitaxel metabolism and paclitaxel-induced neurotoxicity (Article 1)

Paclitaxel exposure has been associated with the severity of the neuropathy (de Graan et al., 2013, Mielke et al., 2005), suggesting that alterations in the activity of paclitaxel metabolizing enzymes and transporters (e.g. CYP2C8, CYP3A4, OATP1B3, OATP1B1 and P-glycoprotein) that result in decreased drug elimination could increase toxicity risk (Rodriguez-Antona, 2010, van de Steeg et al., 2013, Sparreboom et al., 1997). Indeed, the associations between polymorphisms in taxane pharmacokinetic pathways and the development of peripheral neuropathy were the first to be studied.

Concerning CYP2C8, *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* have been shown to produce different proteins with altered enzyme activity (Singh et al., 2008, Dai et al., 2001). *CYP2C8*3* (rs11572080 and rs10509681, in total linkage disequilibrium (LD)) is a frequent allele in the Caucasian population (12%) that encodes for a protein with Arg139Lys and Lys399Arg substitutions (CYP2C8.3). The association of *CYP2C8*3* allele with the risk of paclitaxel-induced neuropathy has been previously evaluated. Heterologous expression of CYP2C8.3 in bacteria and human cell lines indicated a decreased paclitaxel metabolism (Soyama et al., 2001, Dai et al., 2001, Rowbotham et al., 2010, Bahadur et al., 2002). In patients, *CYP2C8*3* allele has been associated with high risk of paclitaxel induced neuropathy in some studies (Leskelä et al., 2011, Hertz et al., 2013, Hertz et al., 2014, Green et al., 2009), but not in others (Abraham et al., 2014, Marsh et al., 2007, Ofverholm et al., 2010, Bergmann et al., 2012, Bergmann et al., 2011b). Owing to these conflicting data, we designed a comprehensive study in collaboration with Dr. Magnus Ingelman-Sundberg group at Karolinska Institute to in depth characterize the effect of *CYP2C8*3* on paclitaxel both *in vitro* and *in vivo*.

Regarding the *in vitro* experiments, the type of expression system used can affect enzyme activity. In line with this, lower intrinsic paclitaxel clearance was observed when CYP2C8.3 was expressed in *E. coli*, yeast or insect cells systems (Dai et al., 2001, Kaspera et al., 2011, Gao et al., 2010, Yu et al., 2013) compared to CYP2C8.1 (wild type protein). However, in mammalian systems (i.e. human hepatoma cells) CYP2C8.3 and CYP2C8.1 kinetics were similar (Soyama et al., 2001). In our study we used mammalian human embryonic kidney cells and found no differences in paclitaxel metabolism between CYP2C8.1 and CYP2C8.3. The differences found among the heterologous expression systems might be due to dissimilar protein folding or interaction with other proteins that may alter substrate metabolism.

We also tested the enzymatic activity of CYP2C8.3 using an antimalarial agent (amodiaquine) and an antidiabetic drug (rosiglitazone) as substrates. As for paclitaxel, with amodiaquine we did not observe differences in kinetic parameters between wild type CYP2C8.1 and CYP2C8.3 enzymes, although previous *in vitro* studies suggested altered metabolic activity (Kaspera et al., 2011, Parikh et al., 2007).

Concerning rosiglitazone, we found a higher clearance for CYP2C8.3, in agreement with other *in vitro* (Kaspera et al., 2011) and *in vivo* studies (Kirchheiner et al., 2006, Aquilante et al., 2008). These differences in enzyme kinetics depending on the substrate have also been shown for other cytochrome P450s (Oscarson et al., 1997), suggesting that the same allele may have different effects among different drugs.

With reference to human data, a cohort of 343 paclitaxel-treated patients was genotyped for *CYP2C8*3* allele. No association was found for *CYP2C8*3* variant and the risk of neuropathy caused by paclitaxel when the maximum neuropathy grade developed during the treatment was analyzed, in agreement with previous studies (Marsh et al., 2007, Ofverholm et al., 2010, Bergmann et al., 2012, Bergmann et al., 2011b). Nevertheless, as paclitaxel neurotoxicity is dose-dependent, we also analyzed the accumulated paclitaxel dose that produced a clinically relevant neurotoxicity (i.e. grade ≥2), rather than just evaluating the presence or lack of neurotoxicity at the end of the treatment. Cycle by cycle neuropathy data was obtained for 148 patients (56 Spanish from our series and 92 additional Danish individuals (Bergmann et al., 2011a)) and the cumulative dose analysis gave a trend towards increased neuropathy risk for *CYP2C8*3* carriers (HR=1.70, P=0.078). This is in agreement with to the increased risk detected in three independent studies using this analysis (Leskelä et al., 2011, Hertz et al., 2013, Hertz et al., 2014). Conversely, Abraham *et al* (Abraham et al., 2014) when performing the same analyses in larger series of patients detected no significant association for *CYP2C8*3* with taxane-related sensory neuropathy (P=0.14 and P=0.27, for maximum neuropathy and cumulative dose analysis, respectively). These discordances may be due to differences in neuropathy assessment or to the different chemotherapy regimens among the studies. Altogether, these data rule out a strong effect of *CYP2C8*3* on paclitaxel-induced neuropathy, but suggest a low/moderate effect on the toxicity.

To sum up, this work indicates that *CYP2C8*3* allele has a low/moderate effect on neuropathy risk through a mechanism that is not detectable in mammalian heterologous expression systems we used. The low/moderate effect could explain the differences found among the different association studies performed, since a moderate effect could only be detected in well-powered studies (e.g. with large sample size, objective neuropathy assessment, cycle by cycle neuropathy analyses).

2. Follow up study of GWAS top hits and characterization of *EPHA* polymorphisms as neuropathy risk markers (Article 2)

Candidate gene approaches have allowed identifying common polymorphisms associated with paclitaxel neuropathy in genes involved in paclitaxel pharmacokinetic and pharmacodynamic pathways.

However, a large part of the inter-individual variation remains to be explained. Genome-wide studies have recently been used to identify novel genes associated with drug-induced neuropathy. In GWAS, hundreds of thousands of SNPs across the whole genome are genotyped simultaneously, allowing to identify new genes that contribute to the basis of human traits such as drug-induced toxicity (Low et al., 2014, Motsinger-Reif et al., 2013). In a GWAS performed by our group, a strong evidence of association with higher neuropathy was found for several ephrin type-A receptors (EPHA): *EPHA4* (rs17348202, $P=1.0x10^{-6}$), top hit of the analysis; *EPHA6* (rs301927, $P=3.4x10^{-5}$) and *EPHA5* (rs1159057, $P=6.8x10^{-5}$), among the 50 top hits (Leandro-Garcia et al., 2013). Interestingly, in a meta-analysis with the GWAS performed by Baldwin *et al*, a SNP in *EPHA5* (rs7349683), in high LD with rs1159057, passed genome wide significance ($P=1.4x10^{-9}$) and was suggested as a neuropathy risk marker (Baldwin et al., 2012).

We followed up these initial findings in "Article 2" of the Thesis. In this study we used a series of 146 paclitaxel-treated patients with neuropathy graded cycle by cycle (57 from Spain and 89 from a previously described Danish cohort (Bergmann et al., 2011a)), to evaluate the role of SNPs detected as top signals in Leandro-Garcia *et al* GWAS as potential neuropathy markers. SNPs in *EPHAs*, in *XKR4*, in *PIK3IP1* and in *SGCG* were selected for genotyping (Leandro-Garcia et al., 2013). We used the cumulative paclitaxel dose analysis to increase the power to detect effects on neuropathy.

We validated the association of *EPHA5*, *EPHA6* and *EPHA8* variant alleles with high risk of neuropathy, in agreement with the GWAS studies (Baldwin et al., 2012, Leandro-Garcia et al., 2013), with the work from Abraham *et al*, in which a large series of patients was used to evaluate several candidate SNPs (Abraham et al., 2014) and with the recent work by Boora *et al* (Boora et al., 2016). In addition, we also studied the effect of the SNPs when using maximum neuropathy grade analysis. With this approach we were only able to detect a trend for *EPHA6*, meaning that the cumulative dose to grade 2 neuropathy is a more sensitive analysis. Regarding the SNP in *EPHA4*, no significant association was found with neither of the analyses. A possible explanation to this is that with a low allele frequency (5%) we do not have enough statistical power to detect the association.

In conclusion, this study provided an independent confirmation of *EPHA5*-rs7349683, *EPHA6*-rs301927 and *EPHA8*-rs209709 as markers of paclitaxel-induced neuropathy, emphasizing the power of genome-wide techniques to detect new pharmacogenetic markers. These results highlight ephrin receptors as key players in paclitaxel-induced peripheral neuropathy. It is also important to mention that as ephrin receptors are involved in several neuronal functions and in neuronal regeneration after injury repair, they may also be relevant markers of neuropathy for other neurotoxic agents (e.g. bortezomib, vinca alkaloids or cisplatin).

Our next goal was to identify novel genetic factors, and potentially with stronger impact, associated with the neuropathy caused by paclitaxel treatment. To achieve this, we designed next generation sequencing approaches (Article 3 and Article 5).

3. Whole-exome sequencing identifies rare defective variants of paclitaxel dose-limiting neuropathy (Article 3)

Despite the significant progress made by GWAS in the identification of *loci* that contribute to complex traits, for several phenotypes only a small fraction of the observed heritability is explained by the common variants so far identified (Manolio et al., 2009). The development of massively parallel sequencing technologies has transformed the field of human genetics. Next-generation sequencing (NGS) can identify all variation in the genome (e.g. common, rare and structural variants) in a cost-effective manner. Although whole genome sequencing (WGS) offers advantages over whole exome sequencing (WES), the latter is a powerful and economic method to detect novel high-impact alleles relevant for complex traits such as adverse drug reactions.

In addition, one strategy to increase the statistical power of a study is to select phenotypic outliers (individuals who are at both ends of a phenotype distribution), rather than using non-selected individuals. This is due to the fact that the alleles that contribute to the trait are enriched in the phenotype extremes. Therefore, following this strategy, even with modest sample sizes it may be possible to identify novel candidate genes and/or alleles associated with diseases and drug outcomes (Nebert, 2000, Cirulli and Goldstein, 2010, Zhang et al., 2006, Westlind-Johnsson et al., 2006). The combination of WES coupled with a carefully selection of extreme phenotype individuals (Shendure and Ji, 2008) has proven to be a powerful method to detect low-frequency susceptibility variants (Ng et al., 2010, Gurwitz and McLeod, 2013, Panoutsopoulou et al., 2013).

Taking all this information into consideration, we selected 8 patients with severe neuropathy to perform WES. To identify the severe-phenotype patients, we used not only the severity of the symptoms during treatment, but also modifications of treatment regimen and long-lasting disabilities.

By WES we detected two rare high-impact variants in *CYP3A4* (*CYP3A4*20* and *CYP3A4*25*). *CYP3A4*20* is an INDEL leading to a premature stop codon previously described in one individual with impaired elimination of CYP3A4 substrates (Westlind-Johnsson et al., 2006). For the novel CYP3A4.25 (p.P389S) protein we demonstrated that it had decreased stability and reduced amounts of protein were detected in a HEK293 expression system. An independent cohort of 228 well-characterized paclitaxel treated patients was used to screen for additional variants in *CYP3A4* exons.

Here, we identified three more carriers of *CYP3A4*20*, and two carriers of *CYP3A4* missense variants: CYP3A4.8 with diminished activity (Eiselt et al., 2001) and CYP3A4.27. For this latter, again we characterized the enzymatic activity and detected less protein expression. In total, 3% of the Spanish patients carried *CYP3A4* defective variants, suggesting that these could explain part of CYP3A4 variability and differences in paclitaxel toxicity.

When performing the statistical analyses, we observed an over-representation of defective *CYP3A4* variants in patients with paclitaxel treatment modifications caused by the neuropathy and in those with high-grade paclitaxel-induced neuropathy. Specifically, patients carrying loss of function (LOF) *CYP3A4* variants (i.e. *CYP3A4*20*) had a significantly higher risk of neuropathy and of paclitaxel treatment modifications, when compared with wild-type *CYP3A4* patients ($P=0.042$ and $P=5.8x10^{-3}$, respectively). It is important to highlight that previous reports only identified markers associated with neuropathy grade, but not with neuropathies resulting in objective clinically relevant consequences: treatment modifications (i.e. paclitaxel dose reductions and treatment suspensions; which do not have a subjective interpretation, but are objective events annotated in the clinical records). Carriers of missense variants showed an intermediate phenotype, concordant with a decreased but not abolished CYP3A4 activity. In line with a previous study (de Graan et al., 2013), the allele *CYP3A4*22*, with reduced activity, had a tendency towards increased paclitaxel treatment modifications. All these results support the implication of CYP3A4 in paclitaxel-induced neuropathy.

In conclusion, applying WES to patients with extreme neuropathy we identified for the first time a marker associated with paclitaxel dose-limiting neuropathy and that may provide a basis for paclitaxel treatment individualization. This study emphasizes the need to screen for rare genetic variants in selected cohorts of patients.

4. High frequency and founder effect of the *CYP3A4*20* in the Spanish population classifies CYP3A4 as a polymorphic enzyme (Article 4)

Interestingly, in Article 3 we found four carriers of the *CYP3A4*20* allele in a population of 236 Spanish paclitaxel-treated patients (2% of carriers). *CYP3A4*20* was first discovered in an individual with impaired elimination of CYP3A4 substrates. Functional studies on this allele showed a lack of enzyme activity (Westlind-Johnsson et al., 2006) and the allele was described as a rare event, in agreement with the highly conserved nature of CYP3A4 (Rahmioglu et al., 2011, Ozdemir et al., 2000). Indeed, only three LOF variants have been described in the literature (*CYP3A4*6, CYP3A4*20, CYP3A4*26*). *CYP3A4*20* is the most common defective allele being carried by 0.2% and 0.07% of Europeans and 0.05% and 0.02% of Africans, according to Exome Variant Server and ExAC browser, respectively. Thus, the described allele frequency of this allele differed with the one we found in Article 3 and we decided to follow up this finding and study the origin and distribution of *CYP3A4*20* allele in different populations.

We collected more than 4000 individuals from European, African and Asian populations and genotyped *CYP3A4*20* allele. We found that 1.2% of Spanish individuals (24 out of 1977) carried the *CYP3A4*20* allele, compared to 0.2% in Portugal (1 out of 450). No other carriers were found in the rest of European, African or Asian populations (that accounted a total of 2118 individuals). Focusing on the Spanish population, the frequency of *CYP3A4*20* carriers varied depending on the region. For example, we observed one *CYP3A4*20* carrier every 26 individuals in Castilla y León, one every 33 in Comunidad Valenciana and one every 48 in Extremadura. These results constitute the first proof that *CYP3A4* LOF alleles can be classified as polymorphisms (i.e. with allele frequencies above 1% in some region of Spain) and can affect a large number of individuals, at least in specific populations.

We also conducted a haplotype analysis to investigate if the variant descended from an ancestral mutation or was an independent event. To perform it, we analyzed four microsatellites (i.e. D7S651, D7S2498, D7S2480 and D7S666) and we genotyped four additional SNPs in the *CYP3A* locus (i.e. *CYP3A4*22* (rs35599367), *CYP3A4*1B* (rs2740574), *CYP3A7*2* (rs2257401) and *CYP3A5*3* (rs776746)). Haplotype reconstruction suggested that *CYP3A4*20* appeared in a single haplotype present in 4% of the chromosomes in the Spanish population. It contained the most common Caucasian variants (Ingelman-Sundberg et al., 2007) for the functional *CYP3A* SNPs (i.e. wild type *CYP3A4*22*, *CYP3A4*1B* and *CYP3A7*2* and variant *CYP3A5*3* allele). Since *CYP3A4*20* is independent of the intronic polymorphism *CYP3A4*22*, which has been previously associated with a decreased elimination of CYP3A4 substrates (Wang et al., 2011, Elens et al., 2013b), individuals carrying both of these two alleles may have a highly reduced CYP3A4 activity.

The high frequency of *CYP3A4*20* in Spain, compared to other countries outside the Iberian peninsula, together with a haplotype spanning 700 Kb and shared by all mutation carriers, suggest a recent occurrence of the allele. We estimated that *CYP3A4*20* appeared about 1000 years ago, which is compatible with a single origin of the mutation in Spain, and then spreading to different geographical areas in recent times.

CYP3A4 plays a crucial role in the biotransformation of a wide range of drugs, including many clinical compounds (Klein and Zanger, 2013), and contributes to the metabolism of endogenous substrates (e.g. vitamin D3, arachidonic acid, bile acids and steroid hormones) (Nakamura et al., 2002, Nebert and Russell, 2002). In *Cyp3a* knock-out mice no major phenotype was detected except for drug metabolism impairment (van Herwaarden et al., 2007, Hashimoto et al., 2013), although in female mice transgenic for *CYP3A4* deficient lactation due to reduced serum estradiol levels was detected (Yu et al., 2005). Thus, *CYP3A4*20* heterozygous carriers may show altered response upon treatment with narrow therapeutic index drugs, although not when treated with drugs with wide therapeutic indexes.

The work by Westlind-Johnsson *et al* supports this concept as they identified an individual heterozygous for *CYP3A4*20* with a 6-fold higher exposure to a drug metabolized by CYP3A4 and low systemic midazolam clearance (Westlind-Johnsson et al., 2006). This individual had a Spanish mother. Regarding homozygous carriers of *CYP3A4*20*, they might not have any clinical manifestation but a severe toxicity profile when exposed to drugs metabolized by CYP3A4. This idea is reinforced by the identification of a patient homozygous for *CYP3A4*26* that experienced a severely diminished tacrolimus clearance (Werk et al., 2014).

In conclusion, thanks to a WES strategy in phenotype outliers we identified a defective rare allele relevant for drug metabolism that is population specific. The high frequency of the allele found in the Spanish population demonstrates a polymorphic nature of *CYP3A4* gene. Furthermore, the key role of CYP3A4 in drug metabolism, together with preliminary clinical evidences, support an increased risk of adverse drug reactions in *CYP3A4*20* carriers and suggests the importance of implementing *CYP3A4*20* genotyping in the clinic, at least for the Spanish population.

5. Targeted sequencing reveals low-frequency variants in *EPHA* genes as markers of paclitaxel-induced peripheral neuropathy (Article 5)

As observed in the previous article, defective low frequency coding variants are expected to have stronger effects on neuropathy than common variants (Cirulli and Goldstein, 2010), and may contribute to the neuropathy variability observed in paclitaxel-treated patients.

However, only our group has used WES to study paclitaxel-induced neuropathy (Article 3). Regarding targeted NGS, during the course of this Thesis Beutler *et al* performed the sequencing of CMT genes in paclitaxel treated patients. They found that three non-synonymous recurrent single nucleotide variants in *ARHGEF10* (i.e. rs9657362, rs2294039, and rs17683288) and rare non-synonymous variants in *PRX* were associated with chemotherapy-induced neuropathy (Beutler et al., 2014). To further explore the hypothesis of low frequency coding variants having an impact on paclitaxel-induced neuropathy, we selected 228 patients with extreme-neuropathy phenotype (high versus no/low neuropathy) to perform massive sequencing of candidate genes. We included genes involved in paclitaxel pharmacokinetics (i.e. *CYP2C8, CYP3A4, ABCB1, SLCO1B1* and *SLCO1B3*), four *EPHA* genes in which common variants have been previously associated with paclitaxel-induced neuropathy (i.e. *EPHA4, EPHA5, EPHA6* and *EPHA8*), and 30 CMT genes. The hypothesis for including CMT genes is that these genes may harbor non-pathogenic genetic variants that, while not leading to CMT, may predispose to chemotherapy-induced neuropathy.

Missense and LOF variants with <0.5% minor allele frequency and passing quality filters were analyzed following a gene-based analysis. The results revealed that low frequency missense variants in *EPHA6* were associated with increased paclitaxel-induced neuropathy risk (P=0.041). Indeed, all *EPHA6* variant carriers had high neuropathy and all the variants were located in the ephrin receptor ligand binding domain, suggesting an alteration of the protein function and further supporting the association with neuropathy. Using the same gene-based analysis we detected a similar trend for *EPHA5* and *EPHA8*. Moreover, two of the variants found in *EPHA5* and in *EPHA8* were LOF, both of them corresponding to high neuropathy patients. In total, 15% (19 of 131) of patients in the high neuropathy group carried low frequency non-synonymous coding variants in *EPHA5*/6/8 genes.

We performed validation in an independent non-selected cohort (i.e. with different grades of neuropathy) of 202 paclitaxel-treated patients with cycle by cycle neuropathy data and performed targeted sequencing for *EPHA5*, *EPHA6* and *EPHA8* coding region. Thirteen *EPHA8* variant carriers were identified but only one *EPHA6* and one *EPHA5* carriers were detected. These results suggested that *EPHA6* and *EPHA5* variants (present in 5 out of the 131 patients with high-neuropathy group in the discovery series) are less frequent in an unselected patient population which included many moderate-neuropathy patients (not represented in the discovery set).

Despite this fact, when applying the accumulated paclitaxel dose analysis, low-frequency variants in *EPHA5/6/8* genes were statistically associated with neuropathy (HR=1.96, P=0.028).

In ExAC browser, 0.1% of the European non-Finish individuals are carriers of LOF variants in either *EPHA5*, *EPHA6* or *EPHA8*. Additionally, on more than 100,000 Icelandic individuals, two complete human knockouts for *EPHA5* and one for *EPHA6* were identified (Sulem et al., 2015). While no phenotype has been assigned to these individuals, based on the literature and on our results, a high susceptibility to drug-induced neuropathy would be expected for them.

Of the LOF variants detected, three occurred in CMT genes (*ARGHEF10*, *IKBKAP* and *DHTKD1*). The patients with variants in *ARGHEF10* and *IKBKAP* belonged to the no/low neuropathy group. This is in agreement with the fact that activating mutations in *ARGHEF10* and not LOF variants can cause CMT (Chaya et al., 2011). Concerning *IKBKAP*, it causes CMT following a recessive model, this can be compatible with a lack of neuropathy in our *IKBKAP* heterozygous individual (Dong et al., 2002). Intriguingly, a variant in *DHTKD1* was present in two patients with different neuropathy grades: one patient with high neuropathy (grade 3) and the other with no neuropathy (grade 0). However, recent evidences question the role of *DHTKD1* in CMT disease and may explain the latter finding. Moreover, two patients with high neuropathy were carriers of the *CYP3A4*20* frameshift allele (already described in Article 3) and another one from the same group carried a LOF in *SLCO1B1*.

Additionally, a patient belonging to the no/low neuropathy was carrier of a splicing variant affecting the last exon of *CYP3A4*, but the effect of this variant on the splicing of the gene and how it affects the enzyme function remains to be studied.

Regarding CMT genes, conversely to Beutler *et al* (Beutler et al., 2014) no significant association with paclitaxel-induced neuropathy was found for variants neither in *PRX* nor in *ARHGEF10*. Nevertheless, a trend towards protection was detected for the common variant rs9657362 in *ARHGEF10* (Beutler et al., 2014, Boora et al., 2015). Interestingly, we have observed a tendency of increased neuropathy risk for the CMT genes *SEPT9* and *SH3TC2*, when performing the gene-based analysis. The differences observed among these studies may be related to dissimilarities in neuropathy assessment, in the selection of patients, or in the distribution of low-frequency variants which have been shown to be population-specific. Thus, further validation in large independent series is required.

In this study we detected for the first time, low-frequency variants in *EPHA5*, *EPHA6* and *EPHA8* conferring increased risk to suffer from paclitaxel-induced neuropathy. Moreover, the function of tyrosine kinase ephrin receptors in neural development (Flanagan and Vanderhaeghen, 1998) and in nerve regeneration after damage (Coulthard et al., 2012, Boyd et al., 2014), in combination with the fact that knockout mice for *EPHA4*, *EPHA5*, *EPHA6* and *EPHA8* present different neurological phenotypes, suggest a relevant role for *EPHA5/6/8* genes in peripheral neuropathy caused by paclitaxel and also by other neurotoxic drugs.

6. Paclitaxel-induced peripheral neuropathy as a multifactorial trait

Paclitaxel-induced neuropathy is a multifactorial trait in which genetics (e.g. *EPHA*, *CYP3A4* and *CYP2C8*3* variants), physiopathologic conditions (e.g. diabetes, alcoholism, liver diseases) and other relevant factors (e.g. age, concomitant drugs) play a role (Figure 5). Evidences supporting the importance for genetics derive from initial candidate gene approaches (Leskelä et al., 2011, Hertz et al., 2013, Hertz et al., 2014), GWAS (Baldwin et al., 2012, Leandro-Garcia et al., 2013) and subsequent studies supporting some of the associations (Abraham et al., 2014, Apellániz-Ruiz et al., 2015, Boora et al., 2016).

Thus, some of the genetic risk factors underlying paclitaxel-induced neuropathy have already been identified. The final goal is to integrate all risk factors contributing to paclitaxel-induced neuropathy into a multifactorial model that would allow a personalized paclitaxel treatment, avoiding severe neuropathy that may compromise treatment success and may affect permanently patients' quality of life.

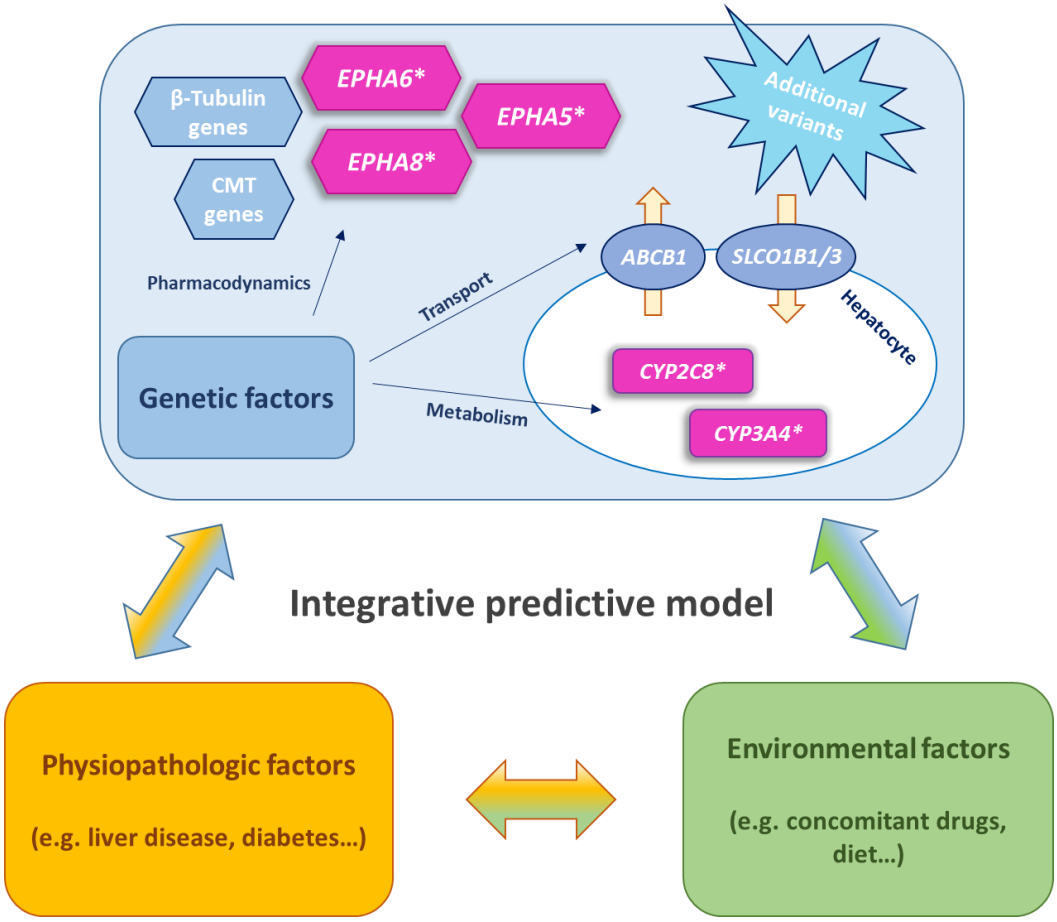


Figure 5. Factors contributing to paclitaxel-induced peripheral neuropathy. Genetic markers highlighted in pink have considerable evidences supporting them to be included in a model to predict peripheral neuropathy risk in patients treated with paclitaxel.

CONCLUSIONS

1. No significant association is found between *CYP2C8*3* allele and the risk of paclitaxel-induced peripheral neuropathy when a maximum neuropathy grade analysis is performed in a cohort of 343 patients. However, a trend towards increased neuropathy is observed when using an accumulated paclitaxel dose analysis. CYP2C8.3 shows a similar enzymatic activity towards paclitaxel than CYP2C8 wild type in a mammalian expression system (HEK293 cell line). Taking into account the results of this study together with previous evidences, we propose that *CYP2C8*3* allele influences paclitaxel-induced neuropathy but with a low/ moderate effect.

2. The common SNPs rs7349683 in *EPHA5*, rs301927 in *EPHA6* and rs209709 in *EPHA8* are confirmed as markers of paclitaxel-induced peripheral neuropathy. The role of EphA receptors in relevant neuronal functions such as nerve repair after injury, suggests that these SNPs could also constitute neuropathy risk markers for other neurotoxic drugs.

3. Defective coding variants in *CYP3A4* increase the risk of paclitaxel-induced peripheral neuropathy and of paclitaxel treatment modifications caused by neuropathy (i.e. dose reductions and treatment suspensions). Thus, *CYP3A4* is identified as an additional predictive marker of paclitaxel-induced neuropathy.

4. The loss-of-function allele *CYP3A4*20* has a high frequency in Spain (with 1.2% of the Spanish population carrying this allele). The fact that *CYP3A4*20* is a rare allele outside Spain and that all *CYP3A4*20* carriers share a common haplotype with low frequency, suggests a Spanish founder effect. The key role of CYP3A4 in drug metabolism suggests an increased risk of adverse drug reactions for *CYP3A4*20* carriers, and supports *CYP3A4*20* genotyping in the Spanish population.

5. Non-synonymous low-frequency variants in the genes *EPHA5*, *EPHA6* and *EPHA8* are associated with increased risk of paclitaxel-induced neuropathy. Interestingly, low-frequency variants in *EPHA6* were present exclusively in patients with high neuropathy and affected the ephrin receptor ligand-binding domain. These results support the use of NGS to detect common and low frequency variants causative of drug adverse events such as paclitaxel peripheral neuropathy.

6. The genetic markers identified in this Thesis may provide the basis for a multifactorial model predictive of paclitaxel neuropathy. This could recognize patients at high risk of developing neuropathy, and provide with a personalized paclitaxel treatment.

CONCLUSIONES

1. No encontramos una asociación significativa entre el alelo *CYP2C8*3* y la neuropatía periférica inducida por paclitaxel cuando analizamos el grado máximo de neuropatía desarrollada en 343 pacientes tratados con paclitaxel. Sin embargo, observamos una tendencia hacia una mayor neuropatía cuando utilizamos un análisis de dosis acumulada. Para el paclitaxel la enzima CYP2C8.3 tiene una actividad enzimática similar al CYP2C8 silvestre en un sistema de expresión de células de mamífero (línea celular HEK293). Teniendo en cuenta los resultados de este estudio y las evidencias previas existentes, proponemos que el alelo *CYP2C8*3* tiene un efecto en la neuropatía causada por paclitaxel pero que este es bajo/ moderado.

2. Confirmamos que las variantes comunes rs7349683 en *EPHA5*, rs301927 en *EPHA6* y rs209709 en *EPHA8* son marcadores de la neuropatía periférica causada por paclitaxel. El papel que desempeñan los receptores de efrinas tipo A en funciones neuronales tales como la reparación de los nervios después de una lesión, sugiere que estos polimorfismos también podrían ser marcadores de riesgo de neuropatías causadas por otros compuestos neurotóxicos.

3. Las variantes codificantes de pérdida de función en *CYP3A4* incrementan el riesgo a desarrollar neuropatía periférica causada por paclitaxel y a tener modificaciones de tratamiento causadas por la neuropatía (reducciones de dosis y suspensiones de tratamiento). Por tanto, el *CYP3A4* es identificado como un marcador predictivo adicional para la neuropatía producida por el paclitaxel.

4. El alelo con pérdida de función *CYP3A4*20* es frecuente en España (1.2% de los españoles porta este alelo). El hecho de que el alelo *CYP3A4*20* sea raro fuera de España y que los portadores de esta variante tengan un mismo haplotipo poco frecuente en la población, sugiere un efecto fundador español. El relevante papel del CYP3A4 en el metabolismo de fármacos sugiere que los portadores del *CYP3A4*20* tienen un mayor riesgo a sufrir efectos adversos, y apoya el genotipado de este alelo en España.

5. Variantes no sinónimas y de baja frecuencia en los genes *EPHA5, EPHA6* y *EPHA8* están asociadas a un mayor riesgo a desarrollar neuropatía inducida por paclitaxel. Es un hecho interesante que todas las variantes de baja frecuencia en *EPHA6* se encontraron en pacientes con alta neuropatía y todas se localizaban en el dominio de unión a ligando. Estos resultados apoyan el uso de NGS para detectar variantes comunes y de baja frecuencia en genes que contribuyen en los efectos adversos causados por fármacos como es el caso de la neuropatía periférica producida por el paclitaxel.

6. Los marcadores genéticos identificados en esta Tesis podrían proporcionar la base para un modelo multifactorial predictivo de la neuropatía. Este podría detectar a los pacientes con mayor riesgo a desarrollar neuropatía, y proporcionar un tratamiento personalizado del paclitaxel.

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APPENDIX: OTHER ARTICLES

Submitted to Breast Cancer Research and Treatment

Polymorphisms associated with everolimus pharmacokinetics, toxicity and survival in metastatic breast cancer.

Tomás Pascual-Martínez*, **María Apellániz-Ruiz*,** Cristina Pernaut, Cecilia Cueto-Felgueroso, Pablo Villalba, Carlos Álvarez, Luis Manso, Lucía Inglada-Pérez, Mercedes Robledo, Cristina Rodríguez-Antona, Eva Ciruelos.

Abstract:

Background: Metastatic breast cancer (MBC) progressing after endocrine therapy frequently activates mTOR pathway. The BOLERO-2 trial showed that everolimus-exemestane achieves increased progression free survival (PFS) compared with exemestane. However, there is great inter-patient variability in toxicity and response to exemestane-everolimus treatment. The objective of this study was to explore the implication of single nucleotide polymorphisms (SNPs) on outcomes from this treatment through a pharmacogenetic analysis. **Patients and Methods:** Blood was collected from 90 postmenopausal women with hormone receptor-positive, HER2-negative MBC treated with exemestane-everolimus following progression after prior treatment with a non-steroidal aromatase inhibitor. Everolimus pharmacokinetics was measured in 37 patients. Twelve SNPs in genes involved in everolimus pharmacokinetics and pharmacodynamics were genotyped and associations assessed with drug plasma levels, clinically relevant toxicities (non-infectious pneumonitis, mucositis, hyperglycemia and hematological toxicities), dose reductions or treatment suspensions due to toxicity, progression free survival (PFS) and overall survival. **Results:** We found that *CYP3A4* rs35599367 variant (*CYP3A4*22* allele) carriers had higher everolimus blood concentration compared to wild type patients (P=0.019). *ABCB1* rs1045642 was associated with risk of mucositis (P=0.031), while *PIK3R1* rs10515074 and *RAPTOR* rs9906827 were associated with hyperglycemia and non-infectious pneumonitis (P=0.016 and 0.024, respectively). Furthermore, *RAPTOR* rs9906827 was associated with PFS (P=0.006). **Conclusions:** *CYP3A4*22* allele influenced plasma concentration of everolimus and several SNPs in mTOR pathway genes were associated with treatment toxicities and prognosis. These results require replication, but suggest that germline variation could influence everolimus outcomes in MBC.

Accepted in JCI Insight

Deep sequencing reveals microRNAs predictive of tyrosine kinase inhibitors response in clear cell renal cell carcinoma patients

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Abstract:

Purpose: The majority of metastatic clear cell renal cell carcinoma (ccRCC) patients are treated with tyrosine kinase inhibitors (TKIs) in first line, however, a fraction are refractory to these drugs. MicroRNAs (miRNA) are regulatory molecules that have proven to be accurate biomarkers in cancer. Here we identified miRNA predictive of progressive disease under TKI treatment.

Patients and methods: Whole miRNA expression was quantified by deep-sequencing in a discovery set of 74 metastatic ccRCC cases uniformly treated with TKIs. MiRNAs differentially expressed in patients progressing under TKI treatment were identified and validated by qRT-PCR in an independent series of 64 patients. Integration of miRNA expression with clinicopathologic factors in a combinatorial model predicted poor response to TKIs.

Results: Twenty nine miRNAs were found to be differentially expressed in the tumors of patients who progressed under TKI therapy (P values from 6x10-9 to 3x10-3). Among six miRNAs selected for validation, an over-expression of miR-1307-3p, miR-155-5p, miR-221-3p, miR-425-5p and miR-222-3p was confirmed in patients with progressive disease as best response (P=4.6x10-3, 6.5x10-3 and 0.034, 0.055 and 0.070, respectively). Furthermore, a 2 miRNA-based classifier could discriminate individuals with progressive disease upon TKI treatment (AUC=0.75, 95%CI=0.64-0.85; P=1.3x10-4) with a better predictive value than clinicopathological risk factors commonly used. miRNAs significantly associated with progression-free survival and overall survival (P=6.8x10-8 and 7.8x10-7 for top hits, respectively) were identified, and 7 miRNAs were found to overlap as predictive for early progressive disease, progression-free survival and overall survival.

Conclusion: This first miRNome comprehensive study demonstrates a predictive value of miRNAs for TKI response and provides a new set of relevant makers that can help rationalize metastatic ccRCC treatment.

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Single nucleotide polymorphism associated with activity and toxicity of cabazitaxel in patients with advanced genitourinary transitional cell carcinoma.

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Abstract:

Aims: We aimed to identify SNPs predictive of cabazitaxel toxicity and response within a phase II clinical trial using this compound in advanced transitional cell carcinoma after progression to a platinum-based regimen.

Methods: Eleven SNPs in *CYP3A4, CYP3A5, CYP2C8, ABCB1* and *TUBB1* were genotyped in 45 patients.

Results: *CYP3A5* rs776746 A-allele was associated with protection against gastrointestinal toxicity (OR=0.06, 95%CI=0.007-0.63, P=0.018) and with reduced progression free survival (HR=5.1, 95%CI=1.7-15.1, P=0.0038, multivariable analysis). *ABCB1* SNPs were associated with total number of grade 3-4 toxicity events (P values of 0.009, 0.041, and 0.043, respectively).

Conclusions: Polymorphisms in *CYP3A5* and *ABCB1* may define a subset of patients with different cabazitaxel toxicity and efficacy and therefore could be used as markers for treatment optimization.

Drug Metabol Personal Ther. 2016 Mar 1; 31(1):3-8

Human genetics: international projects and personalized medicine.

Apellaniz-Ruiz M, Gallego C, Ruiz-Pinto S, Carracedo A, Rodríguez-Antona C.

Abstract:

In this article, we present the progress driven by the recent technological advances and new revolutionary massive sequencing technologies in the field of human genetics. We discuss this knowledge in relation with drug response prediction, from the germline genetic variation compiled in the 1000 Genomes Project or in the Genotype-Tissue Expression project, to the phenome-genome archives, the international cancer projects, such as The Cancer Genome Atlas or the International Cancer Genome Consortium, and the epigenetic variation and its influence in gene expression, including the regulation of drug metabolism. This review is based on the lectures presented by the speakers of the Symposium "Human Genetics: International Projects & New Technologies" from the VII Conference of the Spanish Pharmacogenetics and Pharmacogenomics Society, held on the 20th and 21st of April 2015.

J Med Genet. 2015 Oct; 52(10):647-56.

Recommendations for somatic and germline genetic testing of single pheochromocytoma and paraganglioma based on findings from a series of 329 patients.

Currás-Freixes M, Inglada-Pérez L, Mancikova V, Montero-Conde C, Letón R, Comino-Méndez I, **Apellániz-Ruiz M**, Sánchez-Barroso L, Aguirre Sánchez-Covisa M, Alcázar V, Aller J, Álvarez-Escolá C, Andía-Melero VM, Azriel-Mira S, Calatayud-Gutiérrez M, Díaz JÁ, Díez-Hernández A, Lamas-Oliveira C, Marazuela M, Matias-Guiu X, Meoro-Avilés A, Patiño-García A, Pedrinaci S, Riesco-Eizaguirre G, Sábado-Álvarez C, Sáez-Villaverde R, Sainz de Los Terreros A, Sanz Guadarrama Ó, Sastre-Marcos J, Scolá-Yurrita B, Segura-Huerta Á, Serrano-Corredor Mde L, Villar-Vicente MR, Rodríguez-Antona C, Korpershoek E, Cascón A, Robledo M.

Abstract:

BACKGROUND: Nowadays, 65-80% of pheochromocytoma and paraganglioma (PPGL) cases are explained by germline or somatic mutations in one of 22 genes. Several genetic testing algorithms have been proposed, but they usually exclude sporadic-PPGLs (S-PPGLs) and none include somatic testing. We aimed to genetically characterise S-PPGL cases and propose an evidence-based algorithm for genetic testing, prioritising DNA source. **METHODS:** The study included 329 probands fitting three criteria: single PPGL, no syndromic and no PPGL family history. Germline DNA was tested for point mutations in RET and for both point mutation and gross deletions in VHL, the SDH genes, TMEM127, MAX and FH. 99 tumours from patients negative for germline screening were available and tested for RET, VHL, HRAS, EPAS1, MAX and SDHB. **RESULTS:** Germline mutations were found in 46 (14.0%) patients, being more prevalent in paragangliomas (PGLs) (28.7%) than in pheochromocytomas (PCCs) (4.5%) ($p=6.62\times10(-10)$). Somatic mutations were found in 43% of those tested, being more prevalent in PCCs (48.5%) than in PGLs (32.3%) ($p=0.13$). A quarter of S-PPGLs had a somatic mutation, regardless of age at presentation. Head and neck PGLs (HN-PGLs) and thoracic-PGLs (T-PGLs) more commonly had germline mutations ($p=2.0\times10(-4)$ and $p=0.027$, respectively). Five of the 29 metastatic cases harbored a somatic mutation, one in HRAS. **CONCLUSIONS:** We recommend prioritising testing for germline mutations in patients with HN-PGLs and T-PGLs, and for somatic mutations in those with PCC. Biochemical secretion and SDHB-immunohistochemistry should guide genetic screening in abdominal-PGLs. Paediatric and metastatic cases should not be excluded from somatic screening.

J Mol Med (Berl). 2015 Nov; 93(11):1247-55

Functional and in-silico assessment of MAX variants of unknown significance.

Iñaki Comino-Méndez, Luis J Leandro-García, Guillermo Montoya, Lucía Inglada-Pérez, Aguirre A de Cubas, María Currás-Freixes, Carolyn Tysoe, Rocío Letón, Álvaro Gómez-Graña, Veronika Mancikova, **María Apellániz-Ruiz**, Cristina Rodríguez-Antona, Mercedes Robledo, and Alberto Cascón.

Abstract:
The presence of germline mutations affecting the MYC-associated protein X (MAX) gene has recently been identified as one of the now 11 major genetic predisposition factors for the development of hereditary pheochromocytoma and/or paraganglioma. Little is known regarding how missense variants of unknown significance (VUS) in MAX affect its pivotal role in the regulation of the MYC/MAX/MXD axis. In the present study, we propose a consensus computational prediction based on five "state-of-the-art" algorithms. We also describe a PC12-based functional assay to assess the effects that 12 MAX VUS may have on MYC's E-box transcriptional activation. For all but two of these 12 VUS, the functional assay and the consensus computational prediction gave consistent results; we classified seven variants as pathogenic and three as nonpathogenic. The introduction of wild-type MAX cDNA into PC12 cells significantly decreased MYC's ability to bind to canonical E-boxes, while pathogenic MAX proteins were not able to fully repress MYC activity. Further clinical and molecular evaluation of variant carriers corroborated the results obtained with our functional assessment. In the absence of clear heritability, clinical information, and molecular data, consensus computational predictions and functional models are able to correctly classify VUS affecting MAX.
KEY MESSAGES: A functional assay assesses the effects of MAX VUS over MYC transcriptional activity. A consensus computational prediction and the functional assay show high concordance. Variant carriers' clinical and molecular data support the functional assessment.

Ann Oncol. 2015 Sep; 26(9):1987-93.

Pazopanib in pretreated advanced neuroendocrine tumors: a phase II, open-label trial of the Spanish Task Force Group for Neuroendocrine Tumors (GETNE).

Grande E, Capdevila J, Castellano D, Teulé A, Durán I, Fuster J, Sevilla I, Escudero P, Sastre J, García-Donas J, Casanovas O, Earl J, Ortega L, **Apellaniz-Ruiz M**, Rodriguez-Antona C, Alonso-Gordoa T, Díez JJ, Carrato A, García-Carbonero R.

Abstract:

BACKGROUND: The management of advanced neuroendocrine tumors (NETs) has recently changed. We assessed the activity of pazopanib after failure of other systemic treatments in advanced NETs.

METHODS: This was a multicenter, open-label, phase II study evaluating pazopanib as a single agent in advanced NETs (PAZONET study). The clinical benefit rate (CBR) at 6 months was the primary end point. Translational correlation of radiological response and progression-free survival (PFS) with circulating and tissue biomarkers was also evaluated.

RESULTS: A total of 44 patients were enrolled. Twenty-five patients (59.5%) were progression-free at 6 months (4 partial responses, 21 stable diseases) with a median PFS of 9.5 months [95% confidence interval (CI) 4.8-14.1]. The CBR varied according to prior therapy received, with 73%, 60% and 25% in patients treated with prior multitarget inhibitors, prior mTOR inhibitors and both agents, respectively. A nonsignificant increase in PFS was observed in patients presenting lower baseline circulating tumor cell (CTC) counts (9.1 versus 5.8 months; P = 0.22) and in those with decreased levels of soluble-vascular endothelial growth factor receptor-2 (sVEGFR-2) (12.6 versus 9.1 months; P = 0.067). A trend toward reduced survival was documented in patients with VEGFR3 rs307821 and rs307826 missense polymorphisms [hazard ratio (HR): 12.3; 95% CI 1.09-139.2; P = 0.042 and HR: 6.9; 95% CI 0.96-49.9; P = 0.055, respectively].

CONCLUSIONS: Pazopanib showed clinical activity in patients with advanced NETs regardless of previous treatments. Additionally, CTCs, soluble-s VEFGR-2 and VEGFR3 gene polymorphisms constitute potential biomarkers for selecting patients for pazopanib (NCT01280201).

J Natl Cancer Inst. 2015 Mar 11; 107(5)

Whole-exome sequencing identifies MDH2 as a new familial paraganglioma gene.

Cascón A, Comino-Méndez I, Currás-Freixes M, de Cubas AA, Contreras L, Richter S, Peitzsch M, Mancikova V, Inglada-Pérez L, Pérez-Barrios A, Calatayud M, Azriel S, Villar-Vicente R, Aller J, Setién F, Moran S, Garcia JF, Río-Machín A, León R, Gómez-Graña Á, **Apellániz-Ruiz M**, Roncador G, Esteller M, Rodríguez-Antona C, Satrústegui J, Eisenhofer G, Urioste M, Robledo M.

Abstract:

Disruption of the Krebs cycle is a hallmark of cancer. IDH1 and IDH2 mutations are found in many neoplasms, and germline alterations in SDH genes and FH predispose to pheochromocytoma/ paraganglioma and other cancers. We describe a paraganglioma family carrying a germline mutation in MDH2, which encodes a Krebs cycle enzyme. Whole-exome sequencing was applied to tumor DNA obtained from a man age 55 years diagnosed with multiple malignant paragangliomas. Data were analyzed with the two-sided Student's t and Mann-Whitney U tests with Bonferroni correction for multiple comparisons. Between six- and 14-fold lower levels of MDH2 expression were observed in MDH2-mutated tumors compared with control patients. Knockdown (KD) of MDH2 in HeLa cells by shRNA triggered the accumulation of both malate (mean ± SD: wild-type [WT] = 1±0.18; KD = 2.24±0.17, P = .043) and fumarate (WT = 1±0.06; KD = 2.6±0.25, P = .033), which was reversed by transient introduction of WT MDH2 cDNA. Segregation of the mutation with disease and absence of MDH2 in mutated tumors revealed MDH2 as a novel pheochromocytoma/paraganglioma susceptibility gene.

Breast Cancer Res Treat. 2015 Jan; 149(2):385-94.

Impact of chemotherapy on telomere length in sporadic and familial breast cancer patients.

Benitez-Buelga C, Sanchez-Barroso L, Gallardo M, **Apellániz-Ruiz M**, Inglada-Pérez L, Yanowski K, Carrillo J, Garcia-Estevez L, Calvo I, Perona R, Urioste M, Osorio A, Blasco MA, Rodriguez-Antona C, Benitez J.

Abstract:

Recently, we observed that telomeres of BRCA1/2 mutation carriers were shorter than those of controls or sporadic breast cancer patients, suggesting that mutations in these genes might be responsible for this event. Given the contradictory results reported in the literature, we tested whether other parameters, such as chemotherapy, could be modifying telomere length (TL). We performed a cross-sectional study measuring leukocyte TL of 266 sporadic breasts cancer patients treated with first-line chemotherapy, with a median follow-up of 240 days. Additionally, we performed both cross-sectional and longitudinal studies in a series of 236 familial breast cancer patients that included affected and non-affected BRCA1/2 mutation carriers. We have measured in leukocytes from peripheral blood: the TL, percentage of short telomeres (<3 kb), telomerase activity levels and the annual telomere shortening speed. In sporadic cases we found that chemotherapy exerts a transient telomere shortening effect (around 2 years) that varies depending on the drug combination. In familial cases, only patients receiving treatment were associated with telomere shortening but they recovered normal TL after a period of 2 years. Chemotherapy affects TL and should be considered in the studies that correlate TL with disease susceptibility.

Thyroid. 2014 Aug; 24(8):1251-5

VEGF, VEGFR3, and PDGFRB Protein Expression Is Influenced by RAS Mutations in Medullary Thyroid Carcinoma.

Mancikova V, Inglada-Pérez L, Curras-Freixes M, de Cubas AA, Gómez A, Letón R, Kersten I, Leandro-García LJ, Comino-Méndez I, **Apellaniz-Ruiz M**, Sánchez L, Cascón A, Sastre-Marcos J, García JF, Rodríguez-Antona C, Robledo M.

Abstract:

BACKGROUND: Tyrosine kinase inhibitors (TKIs) have achieved remarkable clinical results in medullary thyroid carcinoma (MTC) patients. However, the considerable variability in patient response to treatment with TKIs remains largely unexplained. There is evidence that it could be due, at least in part, to alterations in genes associated with the disease via their effect on the expression of TKI targets. The objective of this study was to evaluate the influence of RAS mutations on the expression levels in MTC tumors of eight key TKI target proteins.

METHODS: We assessed by immunohistochemistry the expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 in a series of 84 primary MTC tumors that had previously been molecularly characterized, including 14 RAS-positive, 18 RET(M918T)-positive, and 24 RET(C634)-positive tumors, as well as 15 wild-type tumors with no mutations in the RET or RAS genes.

RESULTS: In contrast to RET-positive tumors, RAS-positive tumors expressed neither PDGFRB nor MET (p=0.0060 and 0.047, respectively). Similarly, fewer RAS-positive than RET-related tumors expressed VEGFR3 (p=0.00062). Finally, wild-type tumors expressed VEGF more often than both RAS- and RET-positive tumors (p=0.0082 and 0.011, respectively).

CONCLUSIONS: This is the first study identifying that the expression of TKI targets differs according to the presence of RAS mutations in MTC. This information could potentially be used to select the most beneficial TKI treatment for these patients